Hon. Barbara J. Rothstein

UNITED STATES DISTRICT COURT
WESTERN DISTRICT OF WASHINGTON
AT SEATTLE

ACCESS TO ADVANCED HEALTH INSTITUTE,

Plaintiff,

vs.

PATRICK SOON-SHIONG, M.D. and CHAN SOON-SHIONG FAMILY FOUNDATION,

Defendants.

Case No. 2:24-CV-01253-BJR

DECLARATION OF ALICIA COBB IN SUPPORT OF DEFENDANTS' OPPOSITION TO PLAINTIFF'S EMERGENCY MOTION FOR TEMPORARY RESTRAINING ORDER

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I, Alicia Cobb, declare:

1. I am an attorney licensed to practice in the State of Washington and am admitted to practice before this Court. I am a partner with the law firm Quinn Emanuel Urquhart & Sullivan, LLP, counsel for Defendants Patrick Soon-Shiong, M.D. and Chan Soon-Shiong Family Foundation. I submit this declaration in support of Defendants' Opposition to Plaintiff's Emergency Motion for Temporary Restraining Order, which is being filed concurrently with this declaration. I have personal knowledge of the matters set forth in this declaration, and if called as a witness, I would testify competently to those matters.

2. Attached to the declaration as **Exhibit 1** is a true and correct copy of a journal article titled, "NF-xB activation of the cytomegalovirus enhancer is mediated by a viral transactivator and by T cell stimulation," which was downloaded from https://doi.org/10.1002/j.1460-2075.1989.tb08610.x on August 19, 2024.

3. Attached to the declaration as **Exhibit 2** is a true and correct copy of a journal article titled, "Human cytomegalovirus IE1 transactivates the alpha promoter-enhancer via an 18-base-pair repeat element," which was downloaded from https://www.researchgate.net/publication/20618995 on August 19, 2024.

4. Attached to the declaration as **Exhibit 3** is a true and correct copy of a journal article titled, "Human Cytomegalovirus *ie*2 Negatively Regulates α Gene Expression via a Short Target Sequence Near the Transcription Start Site," which was downloaded from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC239829/pdf/jvirol00045-0341.pdf on August 19, 2024.

5. Attached to the declaration as **Exhibit 4** is a true and correct copy of a journal article titled, "Molecular Genetic Analysis of Cytomegalovirus Gene Regulation in Growth, Persistence and Latency," which was downloaded from https://doi.org/10.1007/978-3-642-74980-3_3 on August 19, 2024.

6. Attached to the declaration as **Exhibit 5** is a true and correct copy of the Curriculum Vitae of Edward S. Mocarski, Jr. Ph.D., which was downloaded from

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https://cap.stanford.edu/profiles/viewCV?facultyId=4146&name=Edward_Mocarski on August 19, 2024.

I declare under penalty of perjury under the laws of the State of Washington that the foregoing is true and correct and that this Declaration was executed on August 21, 2024 at Seattle, Washington.

/s/ Alicia Cobb

Alicia Cobb, WSBA #48685
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CERTIFICATE OF SERVICE

I hereby certify that on August 21, 2024, a true and correct copy of the foregoing was filed electronically by CM/ECF, which caused notice to be sent to all counsel of record.

/s/ Alicia Cobb
Alicia Cobb, WBSA #48685

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Exhibit 1

The EMBO Journal vol.8 no.13 pp.4251 - 4258, 1989

NF-κB activation of the cytomegalovirus enhancer is mediated by a viral transactivator and by T cell stimulation

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Communicated by B.A.D.Stocker

The expression of cytomegalovirus α (immediate early) genes is under control of an enhancer that carries signals for strong constitutive expression as well as response elements for transactivation by viral proteins. We have used synthetic oligonucleotides representing the 16, 18 and 19 bp repeat elements within the enhancer to investigate the role of virus-induced cellular transcription factors in enhancer activation. We show that the transcription factor NF-κB, which binds to the 18 bp repeat, plays a central role in enhancer activation in infected human fibroblasts and that activation is mediated by the product of the viral gene *ie*1. The simian immunodeficiency virus κB site can functionally substitute for the 18 bp element in transient transactivation assays and can also compete efficiently for specific binding to the 18 bp repeat element *in vitro*. Point mutations in the NF-κB site within the 18 bp element disrupt *ie*1-mediated transactivation and binding. We have found that the characteristics of the 18 bp binding factor from human fibroblasts are indistinguishable from NF-κB induced by phorbol ester plus mitogen treatment of T lymphocytes, as determined by gel mobility shift assay as well as protection of the binding site from chemical cleavage. Furthermore, T cell stimulation mediates activation of the viral enhancer via κB sites, an observation that may be important in the interaction of cytomegalovirus with the naturally infected human host. Thus, NF-κB plays a central role as a target for enhancer activation via viral and cellular factors.

Key words: gene expression/herpes virus enhancer/transactivation

Introduction

Many animal viruses make use of cellular transcription factors for the control of viral gene expression. Human cytomegalovirus (CMV) α (immediate early) genes are under the control of an enhancer that consists of a mixed series of repeated 16, 18, 19 and 21 bp sequence elements (Thomsen *et al.*, 1984; Akrigg *et al.*, 1985; Boshart *et al.*, 1985), as illustrated in Figure 1. Certain of these repeat elements contain binding sites for known cellular transcription factors such as the cAMP responsive element binding factor (CREB, also known as ATF) within the 19 bp repeat element and nuclear factor κB (NF-κB) and activator protein-1 (AP1) within the 18 bp repeat element (Akrigg *et al.*, 1985; Hennighausen and Fleckenstein, 1986; Montminy *et al.*, 1986; Sen and Baltimore, 1986a,b; Ghazal *et al.*, 1987; Lee *et al.*, 1987; Hai *et al.*, 1988). All three of these factors have been shown to mediate activation of cellular gene expression. The presence of recognition sequences for these factors within the CMV enhancer suggests that the virus may use them to trigger or modulate α gene expression in response to viral or cellular inducers.

The CMV α promoter-enhancer is constitutively strong in uninfected human fibroblast (HF) cells and is subject to both positive and negative regulation during virus infection (Boshart *et al.*, 1985; Spaete and Mocarski, 1985; Stinski and Roehr, 1985; Pizzorno *et al.*, 1988). The most important enhancer element for strong constitutive expression appears to be the 19 bp repeat element (Boshart *et al.*, 1985; Stinski and Roehr, 1985; Jeang *et al.*, 1987); however, the participation of specific cellular factors in constitutive expression has not been well documented. Although HeLa cell nuclear extracts contain enhancer binding proteins and can support *in vitro* transcription of the α promoter (Thomsen *et al.*, 1984; Hennighausen and Fleckenstein, 1986; Ghazal *et al.*, 1987, 1988) these cells do not support CMV growth or a high level of enhancer activity. Thus, the significance of enhancer studies performed in such non-permissive cell types is limited. In HF cells, CMV infection activates the enhancer. Virion proteins as well as α proteins appear to be involved in this process (Spaete and Mocarski, 1985; Stinski and Roehr, 1985; Pizzorno *et al.*, 1988; Cherrington and Mocarski, 1989). Transient expression experiments have demonstrated that the product of α gene *ie*1 participates in enhancer activation and acts via the 18 bp repeat element (Cherrington and Mocarski, 1989). We show here that this activation appears to be mediated by a virus-induced cellular factor with the characteristics of NF-κB.

NF-κB plays a role as a mediator of inducible gene expression. Originally found in B cells binding to the κ light chain enhancer (Queen and Baltimore, 1983; Sen and Baltimore, 1986a; Atchison and Perry, 1987), this or a related transcription factor appears to be involved in the induction of a variety of cellular and viral genes (reviewed in Lenardo and Baltimore, 1989), including those encoding interleukin-2 (Ballard *et al.*, 1988; Nabel *et al.*, 1988), interleukin-2 receptor α (Durand *et al.*, 1988; Leung and Nabel, 1988; Ruben *et al.*, 1988), β-interferon (Lenardo *et al.*, 1989; Visvanathan and Goodbourn, 1989), major histocompatibility class I products (Baldwin and Sharp, 1987, 1988) and human immunodeficiency virus (Nabel and Baltimore, 1987).

Here we demonstrate the induction of cellular transcription factors following CMV infection and investigate their role in the activation of the enhancer in HF cells, which permit CMV growth, as well as in Jurkat cells, a non-permissive T cell line. In addition to showing that uninfected HF cells contain proteins that bind constitutively to enhancer repeat

elements, we demonstrate that CMV infection induces at least three factors, corresponding to NF-κB, AP-1 and ATF, as determined by gel mobility shift assays. The induction of NF-κB is the most dramatic change in the pattern of enhancer binding proteins and occurs immediately after infection. A similar activity is induced in T cells by phorbol ester plus mitogen (PMA-PHA) treatment. These studies show that, NF-κB plays a central role in the activation of the enhancer in both human fibroblasts and stimulated T lymphocytes.

Results

CMV infection induces HF cell proteins specific for enhancer repeat elements

We investigated the binding of HF cell nuclear proteins to synthetic copies of the 16, 18 (with and without an internal AP-1 site) and 19 bp repeat elements using the gel mobility shift assay (Fried and Crothers, 1981). The double-stranded oligonucleotide probes used in this analysis represented the sequences of the most promoter proximal copies of the 16 bp (16R) and 18 bp (18R) repeats, the second copy of the 19 bp (19R) repeat upstream from the start site of transcription and the only 18 bp element containing an AP-1 site (AP18) as depicted in Figure 1. All of these elements formed sequence-specific DNA−protein complexes using nuclear extracts from either uninfected or CMV-infected HF cells. Although we did not observe any CMV-induced 16R complexes (data not shown), CMV-infected cell extracts formed 18R-, AP18- and 19R-specific complexes that were not observed with uninfected cell extracts (Figure 2).

The characteristics of three different CMV-induced complexes were analyzed. The most dramatically induced complex was specific for a sequence in common between 18R and AP18 probes (compare lanes 2 and 5 of Figure 2A and B). Both 18R and AP18 carry consensus κB sites (CGGGACTTTCC and GGGGATTTCC respectively). The CMV-induced 18R complex (black arrowhead) was competed by 10- or 100-fold molar excess of 18R or AP18 (Figure 2A, lanes 6,7 and 10,11) and the similar mobility AP18 complex was likewise competed by either oligonucleotide (Figure 2B, lanes 6,7 and 10,11). Neither 19R nor 16R competed for binding of this complex, demonstrating its specificity (compare lanes 14,15 and 18,19 of Figure 2A and B). CMV infection induced a second, slower mobility AP18 complex (white arrowhead) in addition to the faster mobility complex (black arrowhead) in common between 18R and AP18 (Figure 2B, compare lanes 2 and 5). As expected, this slower mobility complex was competed by a 10- or 100-fold molar excess of AP18; however, it was not competed with 18R or 16R (Figure 2B, lanes 10,11,18,19). The 19R probe competed for binding of this slower mobility complex at 100- but not at 10-fold molar excess (Figure 2B, lanes 14,15), consistent with the presence of a CREB/ATF binding site (TGACGTCAA) which is homologous to an AP-1 binding site (TGACTCA). These two transcription factors have been reported to exhibit similar DNA binding specificity as well as antigenic cross-reactivity (Hai *et al.*, 1988). Therefore, the slower mobility complex was likely to be the result of AP-1 binding. Finally, a 19R complex was induced by CMV infection and appeared to replace a more slowly migrating complex detected in uninfected cell extracts (Figure 2C, compare lanes 2 and 5). Competition by 10- or 100-fold molar excess of 19R (Figure 2C, lanes 3,4 and 6,7), but not AP18, 18R or 16R

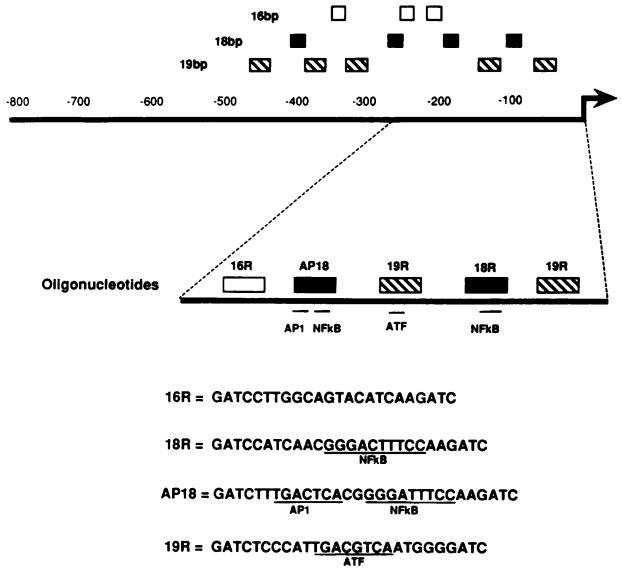

Fig. 1. Structure of the human CMV α promoter-enhancer. The positions of the 16, 18 and 19 bp repeat elements and the single AP18 element are indicated. The site of transcription initiation is marked by an arrow. The region from +1 to −240 relative to the start of transcription is expanded to indicate the location of the elements represented by the synthetic oligonucleotides used in these studies. The sequences of only one strand of the oligonucleotides are shown below. Consensus binding sites for the cellular transcription factors are indicated: CREB/ATF within 19R, NF-κB within 18R, and NF-κB and AP-1 within AP18.

(Figure 2C, lanes 8−11) established the sequence specificity of the 19R complexes.

Temporal changes in binding to enhancer repeat elements during CMV infection

The CMV-induced 19R complex was detected by 24 h after infection (Figure 3A), reflecting a process occurring at early times of viral infection. The faster mobility complex appeared concomitant with the loss of the slower one, raising the possibility that these may represent alternative forms of the same complex. These changes in 19R binding could be important for regulation of the enhancer at later times of infection.

In contrast, the prominent CMV-induced 18R/AP18 complex was detected by 3 h post-infection and persisted through late times (Figure 3B and C). The complex was detected in either infected or uninfected cells after treatment with the protein synthesis inhibitor cycloheximide. Induction by inhibition of protein synthesis is an established characteristic of NF-κB (Sen and Baltimore, 1986b; Wall *et al.*, 1986). The induction of the 18R binding activity in uninfected cells by cycloheximide suggested that the CMV-induced factor was a cellular, rather than viral protein. Because the factor was induced by cycloheximide alone, we could not distinguish whether virion or α proteins, newly synthesized after infection, were responsible for the induction of this complex in infected cells. Previous work (Cherrington and Mocarski, 1989) and transient expression experiments described below suggest that an α gene product is involved. Interestingly, the cycloheximide-induced 18R binding activity correlated with the strong positive effect that protein synthesis inhibitors have on CMV α gene expression (Stinski, 1978; Wathen and Stinski, 1982; Spaete and Mocarski, 1985; Stinski and Roehr, 1985). The second AP18 complex, likely

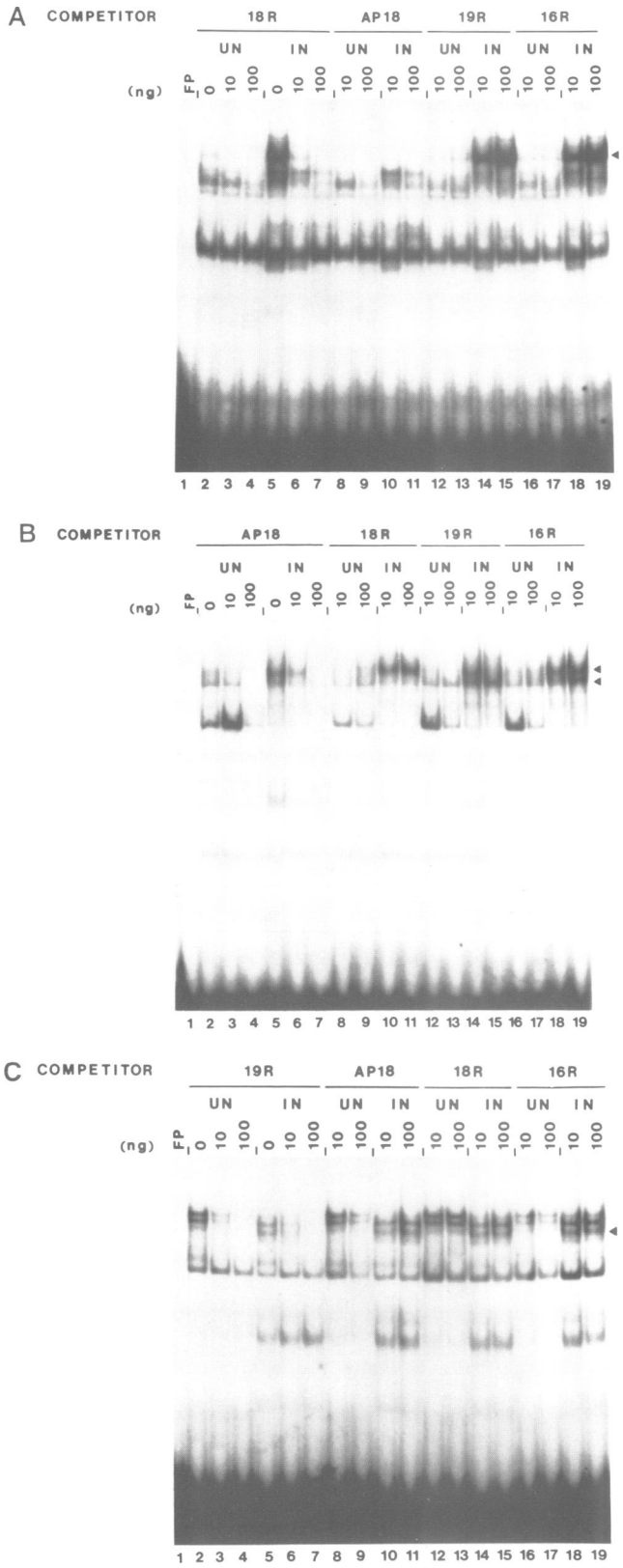

Fig. 2. Mobility shift analysis of CMV-induced binding to 18R, AP18 and 19R probes. Gel mobility shift assays were performed with nuclear extracts from either uninfected HF cells (UN) or HF cells infected with CMV for 48 h (IN). Here and in Figures 3–5 the mobility of the free probe (FP), when no extract is added, is shown in **lane 1**. The arrowhead to the right of the lanes, indicates the CMV-induced complexes. Assays using 1 ng ^{32}P-labeled 18R (**A**), AP18 (**B**) or 19R (**C**) oligonucleotide, incubated without competitor (**lanes 2** and **5**), or with 10 and 100 ng of unlabeled 18R, AP18, 19R or 16R oligonucleotides as indicated (**lanes 3, 4** and **6–19**).

to be due to AP-1 binding, was also induced by 3 h post-infection but was not induced in either infected (Figure 3C) or uninfected (data not shown) cells during cycloheximide treatment. Transactivation of the α promoter occurs immediately after infection and can be detected when protein synthesis is blocked by cycloheximide (Spaete and Mocarski, 1985; Stinski and Roehr, 1985). In addition, transactivation of the enhancer by the *ie*1 gene product occurs via the 18 bp repeat element (Cherrington and Mocarski, 1989). Thus, the characteristics of induction of 18R/AP18 binding activity correlated with many of the expected properties of a factor mediating α gene activation.

The CMV-induced 18R binding activity has the properties of NF-κB

Given the likely importance of the binding activity common to 18R and AP18 to enhancer regulation, we further compared its characteristics to NF-κB. Native cytoplasmic extracts from uninfected or CMV infected HF cells lack the 18R binding activity found in nuclear extracts. Treatment with sodium deoxycholate (DOC), reported to activate a cytoplasmic precursor of NF-κB from B cells (Baeuerle and Baltimore, 1988a), induced activity in cytosolic fractions of both uninfected and infected HF cells (Figure 3B).

The properties of the 18R binding activity induced by CMV in HF cells were compared to NF-κB derived from PMA-PHA stimulated Jurkat cells. This treatment has been observed to produce a rapid induction of NF-κB activity (Sen and Baltimore, 1986b). CMV-infected HF cell nuclear extracts (48 h post-infection) were compared to PMA-PHA stimulated Jurkat nuclear extracts. PMA-PHA treatment of Jurkat cells activated a factor that bound to 18R DNA, had the same apparent mobility as the CMV-induced 18R complex (Figure 4, lanes 2 and 11) and was similarly competed by 18R. Furthermore, the 18R complex formed with either Jurkat or infected HF extracts was efficiently competed by the addition of 10- or 100-fold molar excess of a double-stranded oligonucleotide (GCTGAGACAGCA GGGACTTTCCACAAGGGGAT) derived from the simian immunodeficiency virus enhancer (κB) (Figure 4, lanes 5,6 and 14,15). The only significant homology shared by κB and 18R is the NF-κB binding site (which is underlined). Similar to the 18R complex from HF cells, oligonucleotides 19R and 16R did not compete for binding with Jurkat extracts (Figure 4, lanes 7–10 and 16–19). Therefore, the sequence specificity of the PMA-PHA induced 18R binding activity of Jurkat extracts appeared to be the same as the CMV-induced HF cell activity.

To define further the similarities between the HF and Jurkat activities, we investigated the sequence requirements of complex formation by employing a mutant 18R probe (TTT) containing a triple transversion with the κB site (Figure 5). Binding to the TTT mutant probe was reduced as compared to the wild-type 18R, with either CMV-infected HF or PMA-PHA induced Jurkat nuclear extracts (Figure 5). Previous work has clearly demonstrated that mutants altering these three G residues no longer bind NF-κB (Nabel and Baltimore, 1987; Leung and Nabel, 1988). Thus, in all respects the recognition sequence requirements of the 18R binding factor from both PMA-PHA induced Jurkat cells and CMV-induced HF cells factor were consistent with those of NF-κB.

In addition, the pattern of protection from chemical cleavage of the DNA bound by the NF-κB activities from

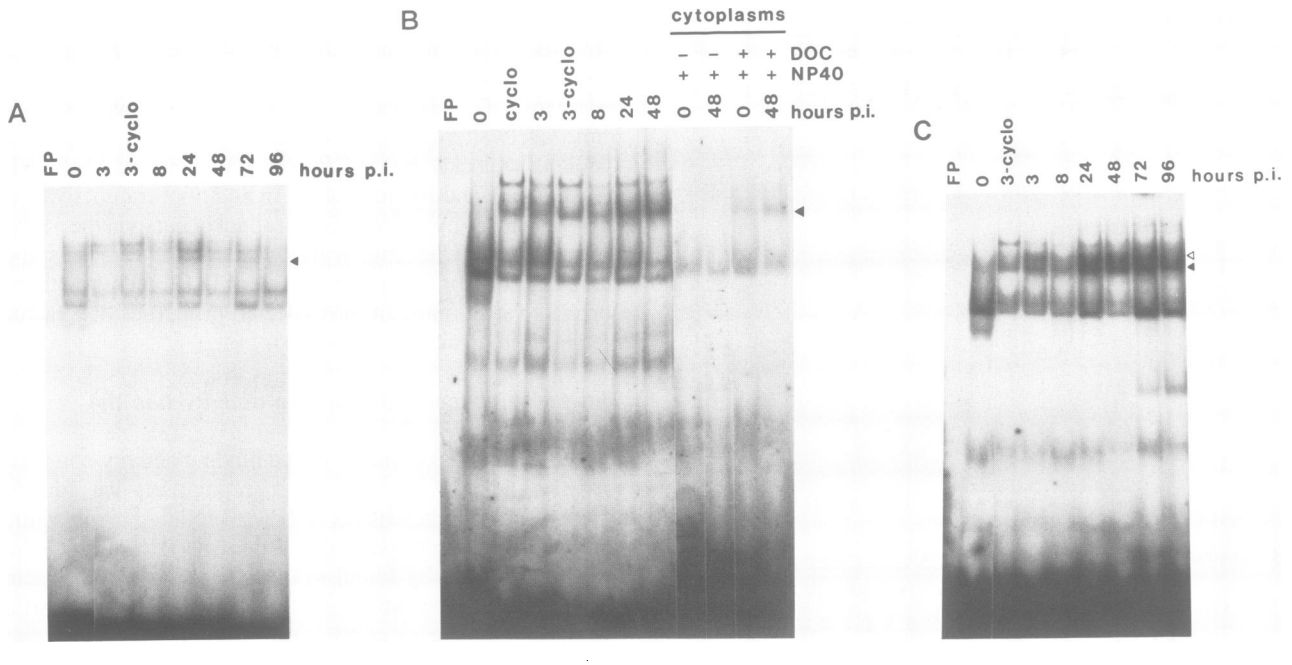

Fig. 3. Changes in binding activity during CMV infection. Gel mobility shift assays were performed with extracts from either uninfected HF cells (0), HF cells treated with cycloheximide (50 mg/ml) for 1 h (cyclo), HF cells treated with cycloheximide from 1 h prior to until 3 h after CMV infection (3-cyclo), or HF cells harvested at either 3, 8, 24, 72 or 96 h after infection with CMV. (**A**) Assay for binding to the ^{32}P-labeled 19R probe with nuclear extracts taken at the indicated times. (**B**) Assays for binding to the ^{32}P-labeled 18R with either nuclear extracts or cytoplasmic extracts (CYTOPLASMS) taken at the indicated times, treated with 1.0% Nonidet P40 alone or in addition to 0.5% sodium deoxycholate. (**C**) Nuclear extracts were assayed for binding to the ^{32}P-labeled AP18 probe. The arrowheads to the right of the lanes indicate CMV-induced complexes as described in the text.

CMV-infected HF and stimulated Jurkat cells were compared. After performing a gel mobility shift assay, the gel was treated with the cleavage reagent 1,10-phenanthroline-copper ion (OP-Cu), and the cleaved DNA extracted from the NF-$\varkappa$B complex. The same region was protected by the HF and Jurkat cell proteins on either strand, demonstrating the identity of these activities (Figure 6).

Activation of CMV α promoter in PMA-PHA stimulated Jurkat cells

The induction of NF-$\varkappa$B binding to the 18 bp enhancer repeat element suggested that activation of CMV α expression should occur in PMA−PHA stimulated Jurkat cells. Transient assays were employed to determine whether the 18 bp element of the CMV enhancer was functionally responsive to NF-$\varkappa$B. Indicator plasmid constructs (Figure 7A), which fused human or murine CMV α promoter-enhancer sequences to the *Escherichia coli lacZ* gene, encoding β-galactosidase, have previously been employed to define the 18 bp element as an important target for autoregulation of enhancer expression by the *ie*1 α gene product (Cherrington and Mocarski, 1989). Jurkat cells were transfected with various indicator plasmid constructs and treated with PMA-PHA 24 h later. β-Galactosidase activity was measured at 42 h after transfection. Jurkat cells transfected with a human CMV promoter-enhancer−*lacZ* fusion construct containing four 18 and five 19 bp human repeats (pON284) produced 39-fold greater β-galactosidase activity after PMA-PHA treatment as compared to equivalent untreated transfectants (Figure 7B). When transfected with the intact murine CMV α promoter-enhancer (pON405; carrying five complete 18 bp elements and one complete 19 bp element), stimulation with PMA-PHA increased β-galactosidase activity 64-fold. In cells transfected with a

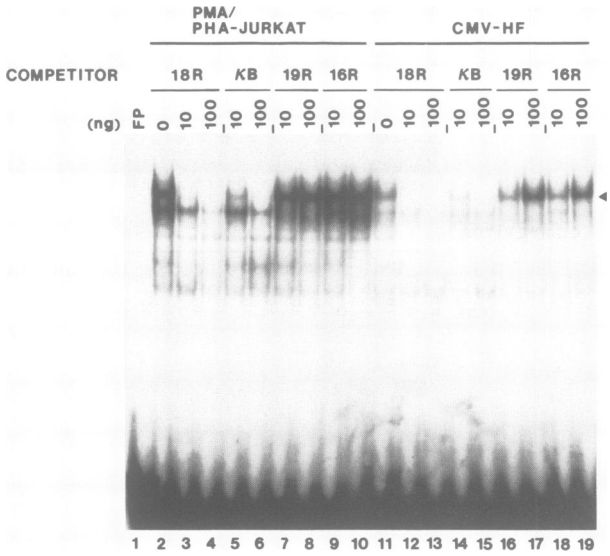

Fig. 4. Comparison of $\varkappa$B binding by extracts of Jurkat or CMV-infected HF cells. Gel mobility shift assays were performed with extracts from either PMA-PHA activated Jurkat cells (PMA/PHA-JURKAT) or HF cells infected with CMV for 48 h (CMV-HF). The arrowhead indicates the CMV and PMA-PHA induced complexes. The binding reaction was performed without oligonucleotide competitor (**lanes 2** and **11**), or with the indicated competitors (**lanes 3−10, 12−19**).

murine CMV α promoter lacking all upstream repeat elements, except for a partial copy of the 19 bp element (pON407), PMA-PHA stimulation resulted in a much lower, 9-fold increase in activity. When three copies of 18R (pON407.18R3) or the SIV-$\varkappa$B (pON407.$\varkappa$BR3) were added back to the murine CMV promoter, PMA-PHA treatment increased β-galactosidase activity 41- and 60-fold respect-

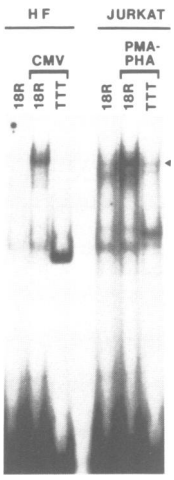

Fig. 5. The effect of a κB mutation on 18R binding. Oligonucleotide TTT was identical to 18R except for a change in the NF-κB site from GGGACTTTCC to TTTACTTTCC. Gel mobility shift assays were performed with mutant and wild-type 18R probes using nuclear extracts from uninfected or 48 h CMV infected HF cells (CMV) and untreated or PMA-PHA induced Jurkat cells (PMA-PHA).

ively over unstimulated levels (Figure 7B). Similar constructs carrying three copies of mutant TTT [pON407(TTT)R3], 19R (pON407.19R3) or 16R (pON407.16R3) were only as responsive as the pON407 control (Figure 7B). The lack of activity of the $18R_{TTT}$ mutant correlated with its inability to bind NF-κB. A single copy of 18R (pON407.18R1) was not sufficient to confer responsiveness to PMA-PHA. Therefore, multiple copies of NF-κB binding sites were necessary for a high level of PMA-PHA activation, indicating a functional role of NF-κB in regulation of the CMV enhancer in T lymphocytes.

CMV ie1 stimulates expression of the enhancer through NF-κB

In HF cells, it has been established that the 18 bp repeat element carries a *cis*-acting signal for transactivation of the CMV enhancer by the *ie*1 gene product, a 491 amino acid protein (Cherrington and Mocarski, 1989). We employed the series of indicator constructs described above to assess whether transcriptional activation by the *ie*1 protein occurred via the NF-κB binding site in the 18 bp repeat. As we have shown previously, HF cells co-transfected with an *ie*1 plasmid along with *lacZ* target constructs carrying either the human or murine CMV α promoter-enhancer, or three copies of 18R added to the murine CMV α promoter (pON407.18R3), showed increased β-galactosidase activity (Cherrington and Mocarski, 1989). Figure 7(C) extends our previous work and shows that the mutant target construct 407(TTT)R3, as well as 407.18R1, were not transactivated by *ie*1. Therefore, multiple NF-κB binding sites were necessary for transactivation by *ie*1, establishing that the *cis* signal for transactivation of α gene expression by *ie*1 in HF cells and PMA-PHA stimulation in T lymphocytes appeared to be an NF-κB binding site.

To assess the role of *ie*1 protein domains in transactivation via NF-κB, we tested two deletion mutants for their ability to transactivate the intact CMV enhancer (pON284). pXD18 carries a deletion of the carboxy-terminal 22 amino acids and pΔAva carries an in-frame deletion between amino acids 24 and 76 of the *ie*1 protein (R.Stenberg, personal communication). Neither of these mutants is able to transactivate the enhancer (Figure 7C); although both produce levels of *ie*1 protein comparable to wild-type (R.Stenberg, personal communication). Therefore, regions near the carboxy- and amino-termini of the protein are important for *ie*1 activation of NF-κB.

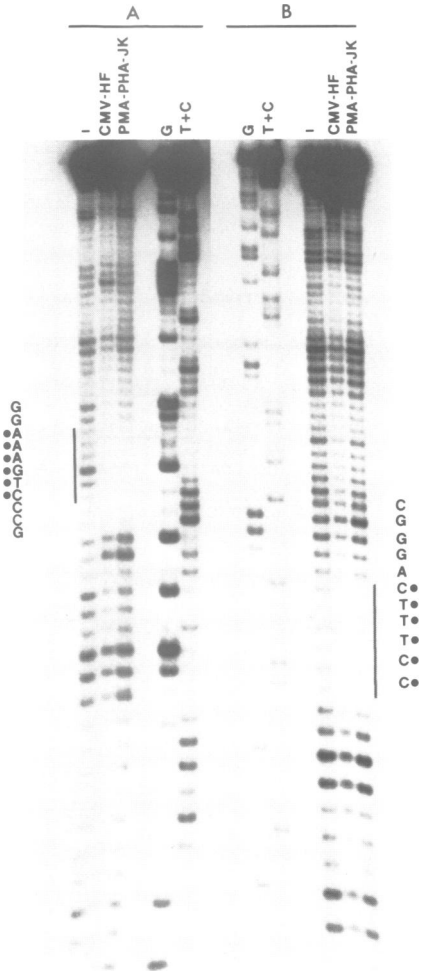

Fig. 6. Protection of the DNA binding site from OP-Cu cleavage of NF-κB proteins from CMV infected HF or PMA-PHA stimulated Jurkat cells. The 18R probe labeled on either the coding strand (**A**) or the non-coding strand (**B**) was used in gel mobility shift assays with nuclear extracts from HF cells infected with CMV for 48 h (CMV-HF) or PMA-PHA stimulated Jurkat cells (PMA-PHA-JK). The cleavage pattern of the free probe, not bound to protein, is shown (−). The DNA sequences protected by the specific complexes are each bordered by a sideline and the protected residues are indicated with dots.

Discussion

NF-κB has been implicated as an inducible transcription factor regulating expression of a variety of cellular and viral genes (Sen and Baltimore, 1986a; Atchison and Perry, 1987; Lenardo *et al.*, 1987; Leung and Nabel, 1988; Nabel and Baltimore, 1988; reviewed in Lenardo and Baltimore, 1989). We have demonstrated that CMV enhancer activation by the *ie*1 gene product in HF cells, as well as by phorbol ester plus mitogen stimulation in T cells, is mediated by a nuclear transcription factor with all the characteristics of NF-κB. These results extend our earlier observation that the 18 bp repeat element within the enhancer carries a target for tran-

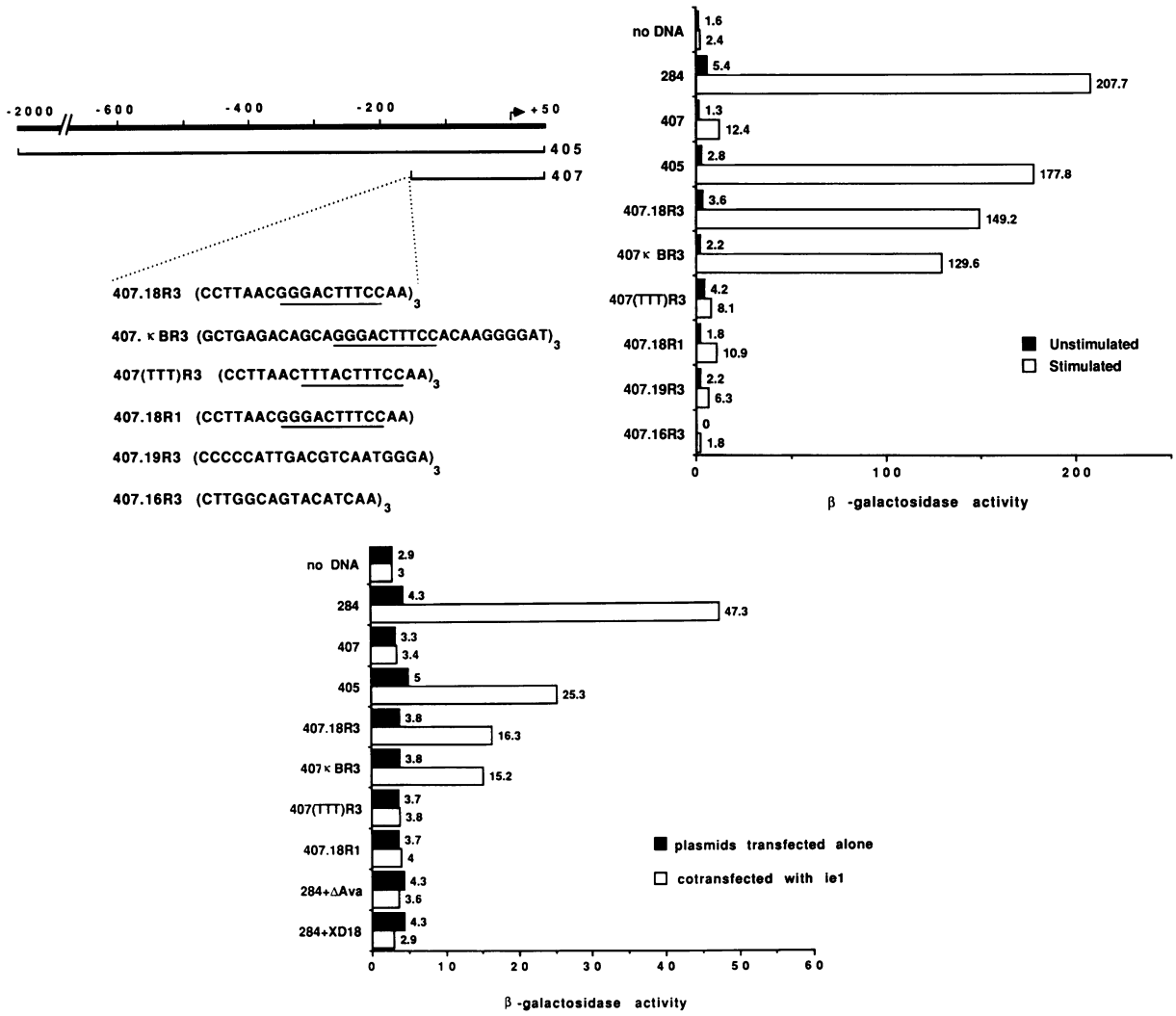

Fig. 7. Activation of CMV promoter constructs in Jurkat and HF cells. Transient assays were performed with constructs derived from the human (284) or murine (405) CMV α promoter-enhancer. (**A**) Synthetic repeat elements from the human CMV α enhancer were added to the murine CMV α promoter (407). The top line represents the intact murine CMV α region from −2000 through +50 relative to the start site of transcription (indicated by an arrow). Three copies of 18R, 19R, 16R, SIV-$\varkappa$B, TTT or a single 18R were inserted just upstream of position −146 of the murine CMV *ie*1 transcription start in pON407 as described (Cherrington and Mocarski, 1989). (**B**) Jurkat cells were transfected with the plasmids described in (A) and were either stimulated with PMA-PHA at 24 h post-transfection (□) or not stimulated (■). β-Galactosidase activity was measured at 48 h post-transfection and is expressed as relative units after subtracting the fluorescence of the medium alone. (**C**) HF cells were transfected with the constructs described in (A) either alone (■) or together with a plasmid carrying the *ie*1 gene (□) (Cherrington and Mocarski, 1989). For analysis of the *ie*1 mutants, 284 was transfected alone (■) or together with mutant ΔAva or XD18 (□). β-Galactosidase activity was measured at 72 h post-transfection as described above.

sactivation by the *ie*1 protein (Cherrington and Mocarski, 1989). Here we show that NF-$\varkappa$B is induced to bind the 18 bp repeat element and is critical for enhancer activation.

We have characterized sequence-specific DNA binding proteins induced by CMV infection that bind to repeated elements of the CMV enhancer, and have shown that the most prominent induced factor corresponds to NF-$\varkappa$B. The properties of the factor induced by CMV in HF cells to bind to the 18 bp repeat element, as defined by competition, mutation and protection analyses, were indistinguishable from those of the NF-$\varkappa$B protein induced by phorbol esters plus mitogen in Jurkat cells. In addition, the temporal characteristics of NF-$\varkappa$B activation during CMV infection were consistent with a role in enhancer regulation at immediate early times. Transient transfection analysis further implicated NF-$\varkappa$B as the factor through which *ie*1 transactivates the CMV enhancer.

Other proteins were induced by CMV to bind to enhancer repeat elements. These include one protein specific for AP18 that might be AP-1 and another specific for 19R that might be CREB/ATF. These induced proteins are also expected to play a role in enhancer regulation, although there is currently no genetic evidence demonstrating their role in enhancer activation. The induced activity specific to the 19 bp repeat appeared by 24 h after CMV infection, suggesting that this factor may participate in regulation of the enhancer at a later stage of the viral life cycle than NF-$\varkappa$B. The sequence-specific DNA binding factors present in uninfected cells may be required for constitutive or regulated expression of the α promoter.

The *ie*1 gene product is a nuclear phosphoprotein (Michelson-Fiske *et al.*, 1977; Tanaka *et al.*, 1979; Gibson, 1983; Stenberg *et al.*, 1984). Although *ie*1 has not been shown to bind DNA directly, it has been recently reported

to associate with metaphase chromosomes (La Femina *et al.*, 1989). Furthermore, analysis of the *ie*1 protein sequence has revealed a strongly acidic carboxy-terminal domain, similar to regions characteristic of a number of viral and cellular transcription factors (Glenn and Ricciardi, 1985; Hope and Struhl, 1986; Lillie *et al.*, 1987; Ma and Ptashne, 1987; Triezenberg *et al.*, 1988; Lillie and Green, 1989). No other clear homologies to structural features of transcription factors have been identified. The carboxy-terminal deletion mutant described here that disrupted *ie*1 activity left intact the acidic domain. Thus, our results do not indicate that the acidic region of the *ie*1 protein functions in a manner analogous to other viral and cellular transcription factors in which similar domains are sufficient for their activity.

The phorbol ester mediated induction of NF-κB is thought to occur through the PKC pathway. The induction has been shown to involve the physical dissociation of a protein inhibitor, IκB, from an inactive cytoplasmic complex (Baeuerle and Baltimore, 1988a,b). Activation of the NF-κB from HF cells also appears to involve conversion of an inactive cytoplasmic precursor to an active nuclear form. Since the CMV *ie*1 gene product stimulates expression of the enhancer through the NF-κB binding site, it is possible that the viral protein promotes dissociation of NF-κB from its inhibitor; however, whether this is accomplished directly, or through intermediates, remains to be established. Preliminary studies with *ie*1 protein synthesized in a reticulocyte lysate system do not support its direct activation of NF-κB (L.C.Sambucetti, unpublished observations). Another possibility that takes into account the known properties of *ie*1 is that it may stimulate expression of NF-κB thereby altering the balance between inactive and active forms of this factor.

The activation of the CMV enhancer in human lymphoblastoid cells may be of physiological importance since this virus can apparently infect these cells and α gene expression is reportedly constitutive during persistence of virus in resting T cells (Rice *et al.*, 1984; Schrier *et al.*, 1985; Braun and Reiser, 1986; Reiser *et al.*, 1986). Furthermore, *in situ* hybridization has detected viral RNA in $CD4^+$ lymphocytes from naturally infected individuals (Schrier *et al.*, 1985). The balance between viral replication and latency in T cells or other tissues may be influenced by NF-κB levels which are, in turn, under control of signals that induce immune cells to respond to antigen. This transcription factor may contribute to viral reactivation during antigen stimulation, as has been proposed for human immunodeficiency virus (HIV; Nabel and Baltimore, 1987). In addition, the induction of this factor in HF cells by the *ie*1 gene product may lead to the activation of cellular genes important for viral growth, to alterations in the immune status of the infected individual or to activation of other viruses residing in the same cells.

Several other animal viruses either have NF-κB binding sites or have been reported to activate NF-κB-like factors. The SV40 and HIV enhancers contain NF-κB recognition sequences (Sassone-Corsi *et al.*, 1985; Nabel and Baltimore, 1987). Hepatitis B virus (HBV) activates an NF-κB-like factor, although the virus has no apparent NF-κB binding sites, suggesting that the function of this factor may be to activate cellular genes necessary for HBV growth (Twu *et al.*, 1989). Likewise, human T cell leukemia virus-1 (HTLV-1) *tax* protein stimulates a factor that is related to NF-κB, judging from its binding site homology; however, this factor is distinct in that it is not PMA inducible. Like HBV, HTLV-1 also lacks NF-κB binding sites (Leung and Nabel, 1988). In contrast, HIV has two NF-κB binding sites within its enhancer although HIV proteins are not known to activate this cellular transcription factor (Nabel and Baltimore, 1987). Thus, the *ie*1 effect on NF-κB provides a possible mechanism for CMV activation of HIV replication in co-infected cells, and may play a role in AIDS progression. Of course, other stimuli could activate these viruses and result in the same pathological consequences.

The human CMV enhancer contains four and the HIV enhancer two NF-κB binding sites (Nabel and Baltimore, 1987). There is a single NF-κB binding site in the κ light chain gene enhancer, the IL-2 and IL-2 receptor gene promoters and the β-interferon gene promoter. Each consists of κB together with other transcription factor binding sites. In the case of the β-interferon promoter, NF-κB acts in combination with other transcription factors to determine tissue specific expression. As reported here, the murine CMV enhancer reconstituted with a single NF-κB recognition sequence did not effectively restore PMA-PHA responsiveness or *ie*1 transactivation (Figure 7). Therefore, optimal NF-κB function may require multimerized binding sites or interaction with other factors. Viral enhancers may utilize the co-operative effect of multiple binding sites to achieve a higher level or a wider range of expression.

Materials and methods

Cell culture and viruses

Human foreskin fibroblasts (HF) and CMV (Towne) were grown as described (Spaete and Mocarski, 1985). Jurkat cells, a $CD4^+$ human T cell line, were grown in RPMI 1640 (GIBCO) supplemented with 10% Nu Serum (Collaborative Research, Inc.). For stimulation, the Jurkat cells were incubated with 50 ng/ml of phorbol myristate acetate (PMA, 4-β-phorbol-12β-myristate-nα-acetate) and 4 μg/ml of phytohemagglutinin (PHA) (Sigma), for 12–24 h prior to measurement of β-galactosidase activity or 3–5 h before harvesting for the preparation of extracts.

Transient assays

Plasmids were introduced into HF or Jurkat cells by DEAE–dextran transfection (Queen and Baltimore, 1983; Sodroski *et al.*, 1984; Spaete and Mocarski, 1985). Following transfection, Jurkat cells were divided equally and either stimulated with PMA–PHA or not treated. β-Galactosidase synthesis directed from these plasmids was measured by adding 4-methylumbelliferyl-β-D-galactosidase (Sigma) to the medium between 42 and 48 h post-transfection, after which the cleavage product was measured on a Dynatech Microfluor fluorometer that excites at 365 nm and reads emission at 450 nm (Geballe *et al.*, 1986).

Extract preparation and gel mobility shift assay

HF cells were harvested either uninfected or 3, 8, 24, 48 or 72 h after infection with CMV (Towne). For protein synthesis block, cells were treated with 50 μg/ml of cycloheximide for 1 h prior to and 3 h after CMV infection. Uninfected cells were treated with the same concentration of cycloheximide for 4 h. Nuclear and cytoplasmic extracts were prepared as described (Dignam *et al.*, 1983) and dialyzed against 10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 1.0 mM EDTA, 1.0 mM dithiothreitol, 20% glycerol (binding buffer). Gel mobility shift assays were performed by incubating 10 μg of nuclear or cytoplasmic extract with 1–2 μg of poly(dI.dC)–poly(dI.dC) (Pharmacia Inc.) in binding buffer for 15 min at room temperature. The ^{32}P-labeled DNA (1.0 ng; 5×10^4 c.p.m.) was added and incubated for an additional 15 min. Non-labeled competitors, when used, were added at the same time as the poly(dI.dC)–poly(dI.dC). The samples were then fractionated by electrophoresis through a 5% polyacrylamide gel run in 45 mM Tris base/45 mM boric acid/mM EDTA, pH 8.3. For treatment of cytoplasmic extracts with detergent, 0.5% deoxycholate was added at the start of the assay, and 1% Nonidet P40 was added 15 min prior to addition of the probe.

Footprinting analysis by protection from cleavage by OP-Cu

After performing a gel mobility shift assay with a DNA probe labeled on a single strand, the entire gel was equilibrated with 200 mM Tris, pH 8.0, for 5 min. For treatment of a 1.5 mm gel, the following were added: 20 ml of 2.0 mM 1,10-phenanthroline (OP)/0.45 nM $CuSO_4$ plus 20 ml of 50 mM 3-mercaptopropionic acid (Sigma) for 15 min at room temperature. The reaction was quenched by addition of 20 ml of 28 mM 2,9-dimethyl-OP for 5 min at room temperature (Kuwabara and Sigman, 1987). After rinsing the gel with water, the DNA–protein complex of interest and free probe were located by autoradiography and excised. The DNA was eluted from the gel by overnight incubation at 37°C in 10 mM Tris, pH 8.0, 1 mM EDTA, 300 mM NaCl, followed by ethanol precipitation and fractionation on a 10% sequencing polyacrylamide gel, alongside sequencing reactions for G and T+C residues (Maxam and Gilbert, 1980).

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Exhibit 2

Human cytomegalovirus IE1 transactivates the alpha promoter-enhancer via an 18-base-pair repeat element

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Human Cytomegalovirus ie1 Transactivates the α Promoter-Enhancer via an 18-Base-Pair Repeat Element

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The expression of α (immediate-early) genes of cytomegalovirus is regulated via a complex enhancer that consists of several different repeat elements. We describe here the autoinduction of expression from the α promoter-enhancer by the most abundant α gene product, a 491-amino-acid nuclear phosphoprotein referred to as ie1. We defined the 18-base-pair repeat element within the α enhancer as the signal through which ie1 acts to regulate gene expression. This element contains an NFκB site that may play an important role in ie1 autoregulation. Our analysis, which relied on deletions through the enhancer as well as reconstitution of responsiveness to a promoter with synthetic 18-base-pair repeats, strongly implicated ie1 in the transcriptional transactivation of the α promoter through its enhancer.

Human cytomegalovirus (CMV), one of five human herpesviruses, has a double-stranded linear DNA genome of 230 kilobase pairs (kbp) (Fig. 1A) encoding over 100 gene products which fall into three kinetic classes and are expressed in a cascade fashion (35; E. S. Mocarski, Transfusion Rev., in press). The α (immediate-early) genes are the first to be expressed after infection, require no de novo protein synthesis, and reach maximal transcription rates by 3 to 8 h postinfection. Three α gene regions have been mapped on the CMV genome (1, 18, 37, 41, 42), with one (0.728 to 0.751 map units [13, 37, 42]) much more abundantly expressed than the others (35). This region consists of four distinct genes: 1, 2A, 2B, and 3 (*ie1*, *ie2A*, *ie2B*, and *ie3*) (14), some of which are expressed via differential splicing from a strong promoter-enhancer (3, 13, 33, 34, 39). The most highly expressed of these four is the *ie1* gene, which encodes a 491-amino-acid (aa) nuclear phosphoprotein (11, 20, 33, 38) referred to as the major immediate-early protein. On the basis of its abundant expression and nuclear localization, *ie1* has been suspected to play a role in regulating other viral genes; however, as yet only one of the CMV α genes, *ie2A*, has been clearly implicated as having a regulatory function (13, 25).

The CMV α promoter-enhancer is very active in uninfected cells and contains repeat elements of 16, 18, 19, and 21 bp (Fig. 1B) that play an important role in transcriptional enhancement (3, 36). Human, murine, and simian CMVs all carry *ie1*-like genes that are abundantly expressed from a strong enhancer-promoter (3, 6, 12, 39). Deletion analyses on the human CMV enhancer have suggested that the repeats act in an additive manner and have implicated the 19-bp repeat as an essential element for enhancer-dependent expression in uninfected HeLa cells (3, 36). Sequence-specific DNA-binding proteins that interact with each of these repeat elements have been identified in uninfected HeLa cells (9, 10, 12). By analogy to other systems, these elements could be sites of action for transcription factors and may confer either positive or negative regulatory characteristics on gene expression (for a review, see reference 15). Consistent with a potential role in transcriptional regulation, the 18-bp repeat contains a sequence that is similar to both the NFκB enhancer element of immunoglobulin genes (30) and a human immunodeficiency virus enhancer element (23). The 19-bp repeat element contains a sequence that is similar to the cyclic AMP-responsive element (22). We have been interested in determining the role played by these repeat elements in the transactivation of the *ie1* promoter-enhancer, as well as identifying the proteins involved in α gene activation.

Immediately after infection of cells in culture with CMV, a virion component signals the transactivation of CMV α genes (31, 36), a process that appears to be analogous to the activation of the α genes of herpes simplex virus (HSV) (2, 4, 24, 26). In CMV, the enhancer is required for virion transactivation (W. C. Manning and E. S. Mocarski, unpublished data); however, the specific target sequences within the enhancer region have not yet been defined, nor has the virion component responsible for α gene transactivation been rigorously characterized.

In order to better understand the *cis*- and *trans*-acting factors involved in the regulation of CMV α gene expression, we adopted a strategy, similar to that used to localize the HSV-1 α *trans*-inducing factor (αTIF; [4, 24]), which would enable us to identify regions of the CMV genome that might encode an α *trans*-inducing factor. A series of CMV (Towne) *Xba*I fragment clones representing different regions of the CMV genome were cotransfected (with DEAE-dextran) into human fibroblast cells (31) along with a target indicator plasmid, pON260 (Fig. 1B), which carries 658 bp of the α promoter-enhancer sequence (−14 to −672 relative to the transcription start site) fused to the *lacZ* indicator gene. Expression of β-galactosidase (β-gal) from pON260 was measured by a 4-methylumbelliferyl-β-D-galactoside cleavage assay (8). Another construct, pON249 (Fig. 1B), containing sequences extending further upstream than those in pON260 (to −1144 relative to the transcription start site), was also used as a target in some of the cotransfection experiments. pON260 and pON249 both carried a complete enhancer with all repeat elements, including three 16-bp repeats, four 18-bp repeats, five 19-bp repeats, and two 21-bp repeats. In addition, pON260 and pON249 contained two and four high-affinity nuclear factor 1-binding sites, respectively, upstream of the enhancer (12, 14).

Among the CMV *Xba*I fragment clones used in cotransfection with the α promoter-enhancer target (pON260), only the *Xba*I E fragment, which carried the *ie1*, *ie2A*, *ie2B*, and

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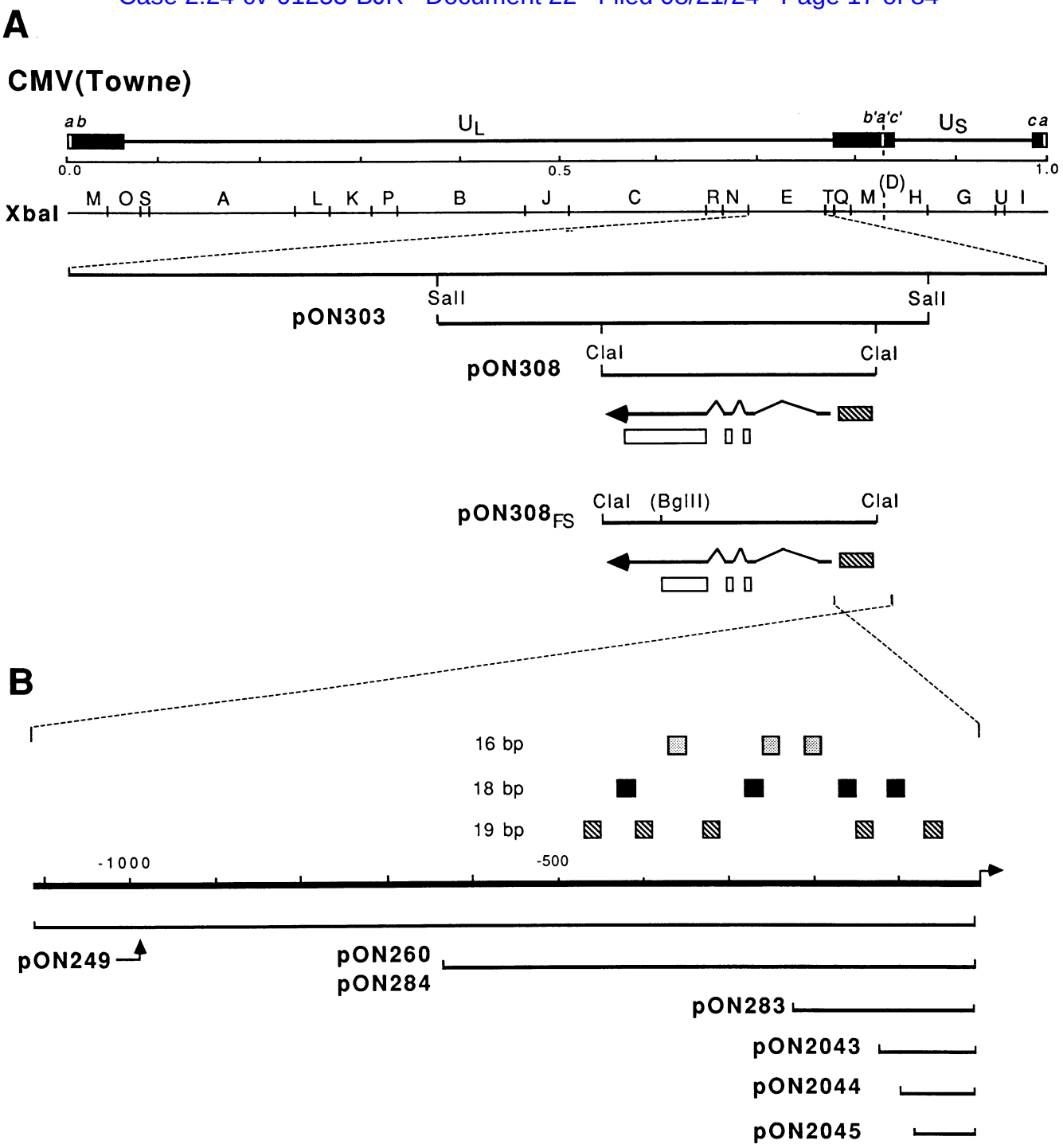

FIG. 1. Organization of the human CMV genome and plasmid constructs used in these experiments. (A) The 230-kbp human CMV genome is depicted on the top line, with the large boxes indicating inverted repeats flanking the unique (U) regions of the L and S components (35). An *Xba*I restriction map is shown below. A *Sal*I fragment cloned in pON303 (31) carrying both the *ie1* and *ie2A* genes and a *Cla*I fragment cloned in pON308 (31) carrying the intact *ie1* gene are depicted in the expanded region. pON303G (see text) is the same *Sal*I fragment and pON308G is the same *Cla*I fragment cloned into pGEM-2 (Promega Biotec). The splicing pattern of the *ie1* gene (four exons and three introns) is depicted along with the protein coding sequences (□) (33). ▨, *ie1* gene enhancer (−118 to −524). ie1 is a 491-aa protein beginning at an initiation codon within the second exon. A frameshift introduced into the unique *Bgl*II site in the fourth exon of *ie1* resulted in the construct pON308$_{FS}$, which is predicted to express a truncated 367-aa ie1. (B) Human CMV α promoter-enhancer regions fused to the *lacZ* indicator gene and used as target constructs in cotransfection experiments. Endpoints of deletions are indicated below and the positions of 16- (▩), 18- (■), and 19- (▧) bp repeat elements are indicated above. pON260 was constructed by deleting the CMV promoter-enhancer sequences between −762 (a *Bal*I site) and −1144 (a polylinker *Sal*I site 6 bp upstream of −1144) in pON249 (8). pON284 carries a CMV DNA fragment identical to that in pON260 but lacks an SV40 origin-enhancer region. pON284 was constructed by cloning a 4.0-kbp *Eco*RI fragment (containing the entire promoter-enhancer and most of the *lacZ* sequences) from pON260 into a 3.0-kbp *Eco*RI fragment from pON3 (containing the carboxyl-terminal sequences of *lacZ*, SV40 polyadenylation signal, and pBR322-based replicon [W. C. Manning and E. S. Mocarski, Virology, in press]). pON283 was created by digesting pON249 with *Bal*1 (sites at −672 and −763 relative to the transcription start site) and subsequent treatment with BAL 31 nuclease (19). pON283 was then linearized at −225 (*Xho*I linker site) and digested with BAL 31 to create pON2043, pON2044, and pON2045. Sizes of deletions were determined by polyacrylamide gel electrophoresis. The precise deletion endpoints, determined by dideoxynucleotide sequence analysis (28), were as follows: pON260 and pON284, −672; pON283, −219; pON2043, −131; pON2044, −103; pON2045, −88 (all relative to the transcription start site).

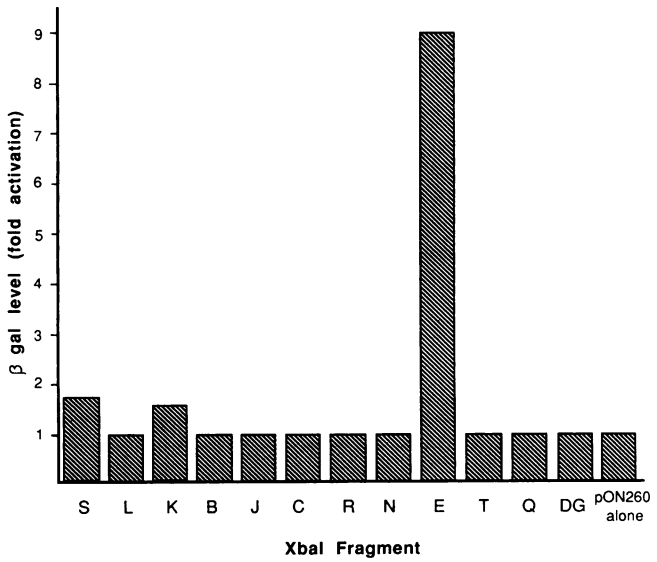

FIG. 2. Survey of human CMV DNA fragments for transactivation of the α promoter-enhancer. The target construct pON260 (10 μg) was cotransfected with individual plasmid clones of CMV (Towne) *Xba*I fragments (20 μg) (40) into human fibroblast cells by using DEAE-dextran (31). Transfected cells were overlaid with Dulbecco minimum essential medium (GIBCO Diagnostics) supplemented with 10% NuSerum (Collaborative Research) containing 150 μg of 4-methylumbelliferyl-β-D-galactoside per ml as described previously (8). 4-Methylumbelliferyl-β-D-galactoside cleavage (β-gal activity) was measured at 48 and 72 h postcotransfection on duplicate dishes. β-gal levels are depicted as fold activation measured after cotransfection with an *Xba*I fragment compared with transfection of pON260 alone, and the values represent an average of β-gal activities measured from 12 separate transfection experiments. The β-gal activities ranged no more than 10 percent above or below the indicated values. Doubling the amount of pON260 DNA (to 20 μg) in the transfection did not detectably increase β-gal levels.

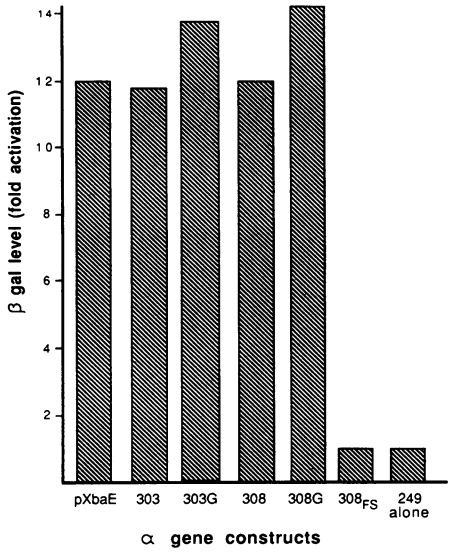

FIG. 3. Transactivation of the α promoter-enhancer required the expression of *ie1*. The target construct pON249 (10 μg) was cotransfected with either constructs carrying α genes or an α gene mutant (20 μg) (Fig. 1) and assayed as described for Fig. 2.

ie3 genes (13, 37), reproducibly transactivated expression of β-gal (Fig. 2). In order to determine which of the α genes within *Xba*I E was responsible for transactivation, we used two previously described (31) plasmids carrying different α gene combinations (pON303 [carrying *ie1* and *ie2A*; Fig. 1A] and pON308 [carrying *ie1*] [Fig. 1A]) in cotransfection experiments. pON303, pON308, and pXbaE transactivated β-gal expression from pON249 to similar levels, suggesting that the function resided in ie1 itself (Fig. 3). As described below, ie1 was able to transactivate the murine CMV α promoter-enhancer (pON405; see Fig. 5); however, target constructs carrying other promoters, including the human CMV $\beta_{2.7}$ promoter (pON241) and the HSV-1 α4 promoter (pON105), were unresponsive to ie1 transactivation (Fig. 4).

To determine whether the effect we observed with pON308 was due to the expression of the *ie1* gene product, we introduced a frameshift mutation at the *Bgl*II site in the fourth exon of *ie1*, resulting in the plasmid construct $pON308_{FS}$ (Fig. 1). This mutation would be expected to frameshift ie1 at aa 345 and result in a premature termination of the protein, reducing its size from 491 to 367 aa. When cotransfected with pON249, $pON308_{FS}$ was inactive. Thus, an intact *ie1* open reading frame (and therefore the expression of *ie1*) was critical for transactivation. To ensure that no other sequences carried by the pON308 construct were acting in concert with *ie1*, the *Cla*I fragment from pON308, which contained the intact *ie1* gene (31, 33), was cloned into a different plasmid vector (pGEM-2 [pON308G]) and shown to be fully capable of transactivating the α promoter-enhancer in transient assays (Fig. 3). The *Sal*I fragment from pON303 containing *ie1* and *ie2A* was also cloned into pGEM-2 (pON303G) and was fully capable of transactivating pON249 (Fig. 3).

pON249 and pON260 both carry a simian virus 40 (SV40) enhancer element 3′ of the *lacZ* gene (8, 31). To determine whether this sequence element was influencing *ie1* transactivation, pON284, which carries an α promoter-regulator region identical to pON260 without the downstream SV40 enhancer, was constructed and found to be identically responsive to ie1 transactivation (Fig. 4). Thus, the presence of the SV40 enhancer did not influence the qualitative or quantitative aspects of *ie1* autoactivation.

To determine the role of CMV enhancer sequence elements responsive to ie1 transactivation, deletion mutants were constructed and used as targets. Plasmids pON249 and pON260 were both significantly transactivated by the CMV fragments carrying *ie1* (Fig. 4). In fact, pON249 was transactivated to a slightly higher level than pON260, which may be a reflection of the presence of two strong nuclear factor 1-binding sites present in pON249. First, we showed that a construct containing a deletion of sequences to −219 bp upstream of the *ie1* transcription start site (pON283) and retaining two copies each of the 18- and 19-bp repeats and one copy of the 16-bp element (but no 21-bp repeats) was transactivated, although to a reduced level (Fig. 4). Next, a series of deletion mutants was generated from pON283 by using BAL 31 nuclease, and each was tested in transient cotransfection assays. A reduction in the level of transactivation by pON308 correlated with removal of 18-bp repeats from the target constructs (Fig. 4). A deletion to −103 bp that partially disrupted the promoter-proximal 18-bp repeat (pON2044) was only slightly transactivated, and a deletion to −88 bp that completely removed this repeat (pON2045) was not stimulated at all. Thus, it appeared that the 18-bp repeat element was an important target for transactivation and that the presence of multiple copies of the element in the α promoter-enhancer was optimal for expression.

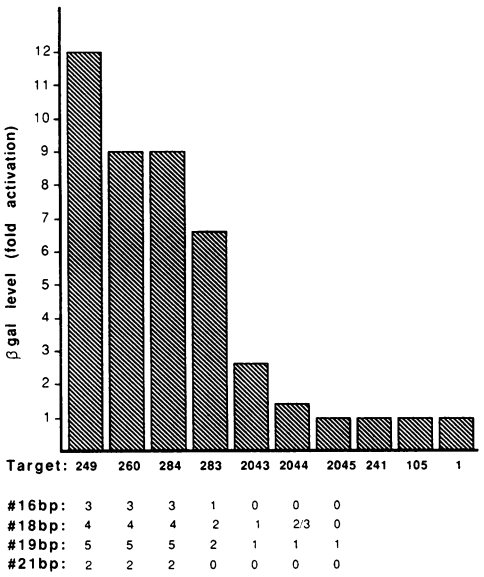

FIG. 4. Transactivation of the α promoter-enhancer by ie1 decreased with the processive removal of enhancer sequences. α promoter-enhancer constructs (10 μg) were cotransfected with pON308 (20 μg) and assayed as described for Fig. 2. Fold activity reflects the level of β-gal activity measured from cotransfection of ie1 and a specific target divided by the level measured from transfection of that specific target plasmid alone. The number of 16-, 18-, 19-, and 21-bp repeat elements carried by each target construct is indicated below the graph. α promoter-enhancer constructs are depicted in Fig. 1; pON241 carries the $\beta_{2.7}$ promoter from human CMV fused to *lacZ* (31), pON105 carries the α4 promoter from HSV-1 (D. Y. Ho and E. S. Mocarski, Virology, in press) fused to *lacZ*, and pON1 lacks a promoter altogether (31). The activities indicated ranged no more than 10 percent above or below the indicated values in replicate experiments.

In order to confirm the observation that ie1 was acting through the 18-bp repeat element to stimulate expression, we performed reconstruction experiments by placing synthetic 16-, 18-, or 19-bp repeats upstream of a promoter devoid of these elements. The promoter we chose to use was derived from the murine CMV α promoter-enhancer, which is responsive to *ie1* transactivation (Fig. 5). The intact murine CMV α promoter-enhancer (pON405) carries five full and six partial 18-bp repeats plus one full and two partial 19-bp repeats (6). Unlike the situation in the human CMV enhancer, the 18- and 19-bp repeat elements in murine CMV are well separated from the Sp1-binding site, CAAT element, and TATA element. Consistent with our studies on the human CMV α promoter-enhancer, a murine CMV α promoter fragment carried by pON407 which contained one partial 19-bp repeat (6) but lacked 18-bp elements altogether was not transactivated by human CMV ie1 (Fig. 5). When three tandem copies of a synthetic 18-bp repeat were added to pON407 (pON407.18R3; Fig. 5A), responsiveness to ie1 transactivation was reconstituted (Fig. 5B). The addition of a single copy of the 18-bp repeat to pON407 resulted in only slight ie1 transactivation (data not shown). As expected from the deletion analysis on the human CMV α promoter-enhancer, pON407-based constructs carrying three copies of either the 19- or 16-bp repeats were not responsive to ie1 transactivation (Fig. 5).

RNA blot analysis was used to determine whether ie1 acted to increase steady-state levels of RNA expressed from the α promoter-enhancer. At 48 h postcotransfection of pON284 with pON308 or pON303, whole-cell RNA was prepared (5), subjected to blot analysis after electrophoretic separation in formaldehyde-agarose gels, and hybridized with a *lacZ*-specific probe (7). As shown in Fig. 6, an increased level of steady-state RNA correlated with the stimulation of β-gal activity from target pON284 upon cotransfection with either *ie1* (lane 3) or *ie1* and *ie2A* (lane 4) compared with the RNA level measured from cells transfected with pON284 alone (lane 1). Lane 2 shows the transcriptional stimulation of pON284 upon superinfection with CMV.

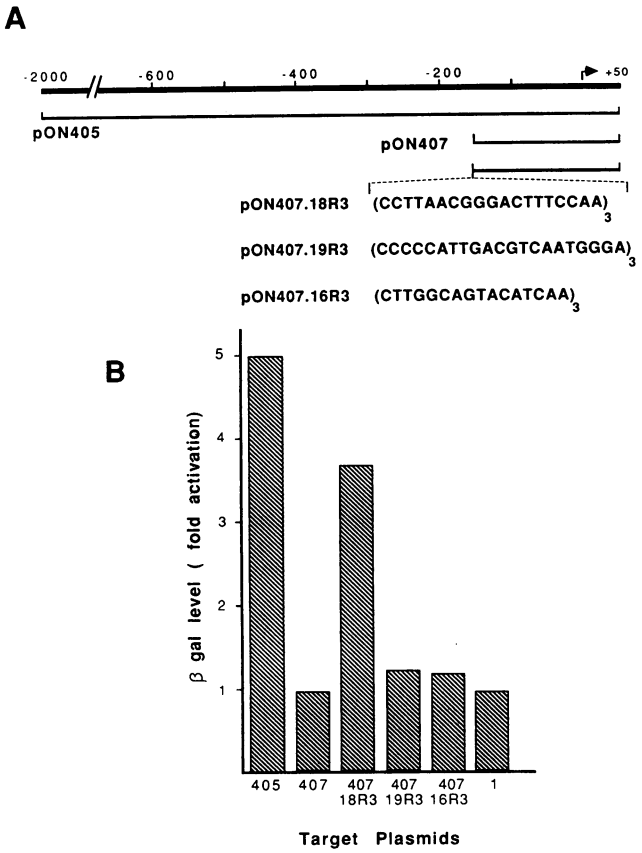

FIG. 5. (A) Murine CMV α promoter and promoter-enhancer constructs used in reconstruction experiments with synthetic human CMV α promoter-enhancer repeat elements. The top line represents the murine CMV α promoter-enhancer region from −2000 through +50 relative to the transcription start site (arrow) (6). pON407 and pON405 have been described elsewhere (Manning and Mocarski, in press). Three tandem copies of the 16-, 18-, and 19-bp repeats (the most promoter-proximal copies of the 16- and 18-bp repeats and the second upstream copy of the 19-bp repeat in the human CMV α promoter-enhancer [3, 39]) were inserted just upstream of position −146 of the murine CMV *ie1* transcription start site in pON407. To construct pON407.16R3, pON407.18R3, and pON407.18R3, pON407 was linearized at a unique *Bam*HI site adjacent to −146, filled in, and then ligated to *Hin*cII-*Sma*I fragments carrying three copies of either the 16-, 18-, or 19-bp repeats (isolated from pTZ3x16R, pTZ3x18R, or pTZ3x19R [G. W. G. Wilkinson, unpublished data], respectively). (B) Synthetic 18-bp repeat elements reconstituted transactivation by ie1. Murine CMV promoter constructs (10 μg) were cotransfected with pON308 (20 μg) and assayed as described for Fig. 2. Fold activity reflects the level of β-gal activity measured from cotransfection of *ie1* and a particular target divided by the level measured from transfection of that specific target alone.

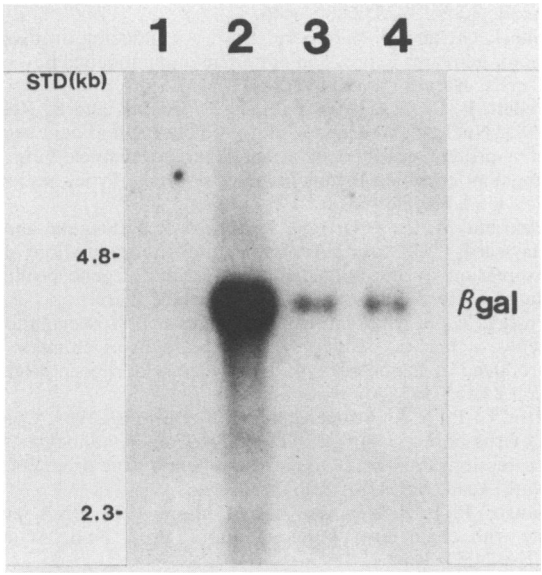

FIG. 6. RNA blot analysis to determine steady-state RNA levels in transfected cells. pON284 (10 μg) was transfected into cells alone (lane 1) or into cells that were subsequently either infected with CMV (10 PFU per cell [8]) (lane 2), cotransfected with pON303 (20 μg) (lane 3), or cotransfected with pON308 (20 μg) (lane 4). RNA was harvested (5) at 48 h postcotransfection and 24 h posttransfection. Whole-cell RNA (10 μg) from each sample was loaded onto a formaldehyde–1% agarose gel, and the RNA was transferred to nitrocellulose paper after electrophoretic separation. The blot was hybridized with a ^{32}P-labeled RNA probe complementary to the 3′-terminal 199 bp of *lacZ*, which detected a 3.6-kilobase *lacZ* transcript (indicated by β-gal to right of lanes). All procedures were as previously described (7, 8), except that a brief RNase (1 μg/ml) treatment of the blot was added before the last wash.

We have demonstrated that the 491-aa ie1 protein is capable of transactivating its own promoter in human fibroblast cells and have localized a critical *cis*-responsive signal to the 18-bp repeat elements that are part of the α enhancer. Although human CMV ie1 is the most abundantly produced α protein, it has not previously been assigned any function in human fibroblasts. Our transient assay results suggest that the function of ie1 is to augment the overall level of expression of α proteins from the α promoter-enhancer or other promoters with the 18-bp target element. Interestingly, the promoter of at least one other human CMV α gene contains the 18-bp element (41), and it will be important to determine whether ie1 transactivation contributes to the regulation of this gene as well. In addition to being active in human fibroblasts, ie1 also transactivated target constructs to similar levels in African green monkey kidney (Vero) cells (data not shown). The effects of ie1 in human fibroblasts or Vero cells are in contrast to its effects in COS-1 cells (African green monkey kidney cells expressing SV40 T antigen), in which ie1 was shown to be autorepressive (32). The reasons for these differences are not clear but may have to do with the added effects of SV40 gene products. Given that our results were obtained in human fibroblasts, which are permissive for CMV growth, we believe our data more closely resemble natural ie1 function, although it is certainly possible that ie1 could naturally exhibit repressing characteristics in some cell types and activating characteristics in others.

It is well established that the α promoter-enhancer has a high basal activity in uninfected cells and that expression is independent of ie1 autoactivation (3, 39). This characteristic of the α promoter-enhancer has led to its use in mammalian cell expression vectors. On the basis of our observations with human fibroblasts as well as with Vero cells, we would expect that expression from this promoter-enhancer could be augmented to even higher levels by the introduction of ie1 in *trans*.

It had been previously demonstrated that the human CMV α promoter-enhancer is transactivated by virus infection even when α gene expression is blocked with cycloheximide (31, 36), suggesting that a virion transactivator analogous to HSV-1 αTIF (4, 24) may be carried by CMV. The ie1 transactivation we describe here is distinct from virion transactivation, but may function in concert with this function (as well as with other transcriptional events occurring through the enhancer) during viral replication.

Besides being expressed immediately after infection in cell culture, the ie1 transcript (or polypeptide) has been detected during persistent infection of human fibroblast cells in culture (21) and during apparent latent infection of human lymphoid cells either taken from seropositive individuals or infected in culture (27, 29). Although our results do not distinguish whether ie1 autoregulation plays a more important role during the productive replication cycle or during latent infection, we speculate that it may be critical in regulating α gene expression during either maintenance of or reactivation from latency. Certainly, much more work is necessary to reveal the physiological setting in which ie1 transactivation is most crucial.

The target for human CMV ie1 transactivation, the 18-bp repeat element, has limited similarity to a core enhancer element from SV40 and is very similar to the NKκB transcription factor-binding site (30) as well as to a related sequence element in the human immunodeficiency virus long terminal repeat (23). ie1 is a nuclear phosphoprotein (11), although it does not appear to be a DNA-binding protein (32). Taken together, these characteristics imply that ie1 may interact with host cell transcription factors, possibly related to NFκB, that bind to the 18-bp element. The occurrence of related *cis*- and *trans*-acting functions in cellular and viral systems suggests they may interact in nature, as has been previously postulated for human immunodeficiency virus (23). Our observations raise the following interesting possibilities concerning the interaction between cells and viruses. (i) CMV ie1 may activate cellular genes that carry the appropriate *cis*-acting elements. (ii) Normal cellular activation of genes with NFκB-like elements may also lead to the activation of CMV gene expression. (iii) CMV ie1 may activate human immunodeficiency virus gene expression via the NFκB-like element under the appropriate circumstances.

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Exhibit 3

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Human Cytomegalovirus *ie2* Negatively Regulates α Gene Expression via a Short Target Sequence Near the Transcription Start Site

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Repression of human cytomegalovirus α (immediate-early) gene expression is under the control of the viral *ie2* gene. Here we show that ie2 negatively regulates gene expression directed by the strong cytomegalovirus enhancer via a specific 15-bp target sequence (which we term *cis* repression signal [*crs*]). This *crs* is located between −14 and +1 relative to the transcription start site and will function in an orientation-independent fashion, consistent with repression occurring at the transcriptional level. Repression is dominant over transactivation by *ie1* gene products. The *crs* (5′-CGTTTAGTGAACCGT-3′) does not contain previously recognized binding sites for cellular transcription factors, and a precise copy is not found elsewhere in the human cytomegalovirus genome. The position of the signal near the transcription start site appears to be important in function; addition of the *crs* near the transcription start site of a heterologous promoter, from the thymidine kinase gene of herpes simplex virus type 1, conferred cytomegalovirus *ie2*-dependent repression upon this promoter. Thus, we propose that an *ie2* gene product or an induced cellular protein mediates repression by binding to *crs*. Negative regulation of α gene expression may be important during viral replication or latency.

Human cytomegalovirus (human herpesvirus 5; CMV), a herpesvirus causing disease primarily in immunocompromised patients, carries over 200 genes whose expression is coordinately regulated and sequentially ordered during viral growth (35, 53). Three regions of α (immediate-early) gene expression have been mapped on the CMV genome (21, 26, 47, 50, 51, 62, 63), with one much more abundantly expressed than the others in infected human fibroblast (HF) cells (10, 21, 59, 61, 63). This prominent α region maps between 0.738 and 0.762 on the CMV genome and includes genes that encode a family of nuclear phosphoproteins (16, 17, 27, 33). Two genes within this region, *ie1* and *ie2*, that carry out regulatory functions (8, 11, 12, 19, 20, 29, 39) are expressed via differential splicing from a single transcription start site (7, 47, 51; Fig. 1). Expression of *ie1* and *ie2* is under control of a strong enhancer (1, 4, 59) and is subject to both positive and negative regulation by factors acting on the promoter-enhancer region (8, 19, 20, 29, 37, 39, 42, 44, 45, 48, 49, 54).

Transient expression experiments have demonstrated that the product of the most abundantly expressed α gene, *ie1*, a 491-amino-acid (aa) protein referred to as the major immediate-early protein (17, 53), participates in enhancer activation via the 18-bp repeat element (8). This positive autoregulation by ie1 is mediated through the cellular transcription factor, NFκB, which binds to a site within the 18-bp repeat element (36, 42). Although *ie1* transcript levels peak between 5 and 8 h postinfection, protein products continue to accumulate throughout infection (47; our unpublished data). One predominant *ie2* gene product 579 aa in size (also called 86K on the basis of electrophoretic mobility) and one less abundant 425 aa in size are expressed immediately after infection from transcripts that are 5′ coterminal and share three exons with *ie1* (47, 51; Fig. 1). At late times, one abundant *ie2* gene product (302 aa in size) is expressed from a promoter that maps just downstream of the *ie1* polyadenylation signal (47, 51). Spliced *ie2* transcripts structurally similar to those expressed immediately after infection continue to persist throughout infection (47).

The transactivating and repressing properties of *ie2* products have been reported. In combination with *ie1*, the *ie2* region is capable of complementing an adenovirus mutant defective in E1a (56, 57). In transient assays, *ie2* gene products can transactivate a variety of heterologous target promoters: herpes simplex virus type 1 (HSV-1) glycoprotein D (12); adenovirus E2 (20, 58); human immunodeficiency virus type 1 long terminal repeat (9); and HSV-1 ICP4 (IE175), simian virus 40 early, adenovirus E3, and β interferon (39). The presence of *ie1* gene products generally increases transactivation of these promoters in the presence of the *ie2* gene, presumably through increasing the levels of expression of *ie2* gene products. Significantly, at least four CMV β (delayed-early) promoters can be transactivated by the *ie2* products in conjunction with *ie1*: the promoter of the β gene encoding the 2.2-kb transcript (46), the promoter of the DNA polymerase gene (11), the promoter of the β gene encoding the predominant 2.7-kb transcript (25), and the multiple promoters of the β gene encoding the 1.7-kb transcript (6). The work of Pizzorno et al. (39) has suggested that important ie2 transactivating functions are contained within the amino-terminal sequences shared between ie1 and either the 579- or 425-aa species of ie2. Further work on the 579-aa protein has implicated both amino- and carboxyl-terminal sequences in transactivation (48).

In addition to the transactivating properties of the *ie2* products, Pizzorno et al. (39) were the first to show that these products may negatively autoregulate the α promoter-enhancer. The target construct that was repressed in their transient expression assays contained CMV α promoter-

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enhancer sequences from −760 to +10 (relative to the transcription start site) fused to an indicator gene. Using effector plasmids with deletions in *ie*1 and *ie*2 in transient cotransfection assays, they demonstrated that the repression function resided in a region distinct from the transactivation domain, i.e., that the repression function, unlike the transactivating function, lies outside of the sequences shared by *ie*1 and *ie*2 gene products, within the *ie*2 gene (exon 5) of the α region. This work has been recently confirmed and extended by others (19, 48) who have shown that a carboxyl-terminal domain of this ie2-specific exon is involved in negative regulation.

In this report, we show that the *cis* target site through which ie2 negatively regulates the α promoter-enhancer is a 15-bp sequence located from −14 to +1 (relative to the transcriptional start site). Repression is dominant over transactivation by *ie*1 gene products. This sequence will function in an orientation-independent fashion, consistent with repression occurring at the transcriptional level. The results predict a direct mechanism for ie2-mediated repression that may involve blocking formation of an active transcription complex.

MATERIALS AND METHODS

Cell culture, viruses, and transient assays. HF cells, Vero cells, and CMV (Towne) were grown as described previously (44). Plasmids were introduced into HF or Vero cells by DEAE-dextran-mediated transfection (40, 43, 44). Following transfection, β-galactosidase (β-gal) synthesis directed from these plasmids was measured by adding 4-methylumbelliferyl-β-D-galactoside (Sigma) to the medium between 48 and 72 h posttransfection, at which time the cleavage product was measured on a Dynatech Microfluor fluorimeter that excites at 365 nm and reads emission at 450 nm (14). All transient assays were performed at least four times, with all critical assays performed at least 10 times. The effector-to-target ratio in our experiments was 2:1, a ratio which does not result in enhancer competition effects (8; data not shown). In most experiment, 10^6 HF cells were transfected with 10 μg of target and 20 μg of effector plasmid.

Plasmid constructs. Plasmids pXbaE, pON308, and pON303 have been described elsewhere (44, 60). pON303ΔAcc, which is similar to plasmids constructed by others (20, 39), was constructed by digesting pON303 with *Acc*I (Fig. 1) and subsequently religating. Indicator plasmids pON249, pON284, pON283, pON2044, and pON2046 have been described previously (8, 14, 44). pON249 contains the α promoter-enhancer sequences from −1138 to −14 fused to *lacZ*. pON284 contains the α promoter-enhancer sequences from −672 to −14 fused to *lacZ*. The 5′ endpoints of the plasmids are as follows: pON284, −672; pON283, −219; pON2044, −103; and pON2046, −75. All of these constructs extend 3′ to −14 (all numbers are relative to the transcription start site). pON239 contains α promoter-enhancer sequences from −1138 to +913 (including the first exon and first intron of the α locus) fused to *lacZ* (44). pON249crs was constructed by digesting pON249 at the unique *Hin*dIII site (polylinker site at −5 relative to the transcription start site) and inserting the double-stranded synthetic −14 to +9 oligonucleotides 5′-AGCTT**CGTTTAGTGAACCGTCAGA TCGCA**-3′ and 5′-AGCTT**GCGATCTGACGGTTCACTAA ACGA**-3′ (CMV DNA sequences shown in bold). Clones of the pON249crs substitution mutation series were all constructed in the same way as pON249crs except that a synthetic double-stranded *cis* repression signal (*crs*) oligonucleotide carrying a specific substitution mutation was inserted in place of the wild-type −14 to +9 sequence (see Fig. 6). pON2200 was constructed by digesting pON249 at the unique *Sna*BI site (at −242) and inserting the wild-type −14 to +9 oligonucleotide after filling in the *Hin*dIII ends with Klenow polymerase (30). pON239*crs*E was constructed by ligating together three fragments to create a plasmid that contained the α promoter-enhancer sequences from −1138 to +1 fused to *lacZ*. The first was a 7-kbp *Hin*dIII-*Sal*I fragment generated from pON239 that contained *lacZ* and pBR322 sequences; the second was an 1,124-bp *Sac*I-*Sal*I fragment from pON239 that contained α promoter-enhancer sequences from −1138 to −14; and the third was double-stranded synthetic oligonucleotide containing the sequences 5′-CGTTTAGTGAACCGTA-3′ and 5′-AGCTTACGGTTC ACTAAACGAGCT-3′ (5′ *Sac*I end and a 3′ *Hin*dIII end) representing α promoter sequences from −14 to +1. The enhancer deletion plasmids were constructed as follows: parent *lacZ* indicator plasmids (8) with various amounts of enhancer sequences, pON283 (containing −219 to −14), pON2044 (containing −103 to −14), and pON2046 (containing −75 to −14), were digested with *Hin*dIII, and the −14 to +9 *crs* oligonucleotide was inserted into the *Hin*dIII site of each plasmid, resulting in pON283crs, pON2044crs, and pON2046crs. pON2201 was constructed by inserting the 240-bp *Pst*I HSV-1 β thymidine kinase (TK) promoter fragment from TK/LS −6/−16 (32) into pON3 (31) which had been digested with *Pst*I. pON2202 was constructed by digesting pON2201 with *Bam*HI, filling in with Klenow enzyme, and ligating to the filled-in double-stranded *crs* oligonucleotide; the sequence was cloned in the natural 5′-to-3′ (−14 to +9) orientation. pRB201 contains the HSV-1 *Hin*dIII HM junction fragment with the HSV-1 α0, α4, and α27 genes.

RNA isolation and blot hybridization. Whole-cell RNA was isolated by the guanidium isothiocyanate method (30). A 10-μg sample of each RNA was electrophoresed on a formaldehyde–1% agarose gel, and the RNA was transferred to nitrocellulose and probed as previously described (8, 13, 14). Equivalent amounts of RNA were loaded on each lane, as indicated by identical rRNA bands following ethidium bromide staining.

Immunofluorescence assays. Transfected HF cells were grown on glass coverslips, and 72 h posttransfection the cells were washed in medium and fixed in cold acetone for 3 to 5 min. They were then incubated with 100 μl of a mixture of mouse monoclonal antibody (MAb810; Chemicon) directed against an epitope encoded by the third exon of the CMV α gene locus (27; Fig. 1) and a polyclonal anti-β-gal antibody (Cappel) at final dilutions of 1:100 and 1:60, respectively, for 30 min at 20°C. After the cultures were washed in medium, the cells were exposed to a mixture of tetramethylrhodamine isothiocyanate (TRITC)-conjugated $F(ab')_2$ fragments of goat anti-mouse immunoglobulin G (IgG) and fluorecein isothiocyanate (FITC)-conjugated goat IgG anti-rabbit IgG (both from Cappel) at final dilutions of 1:100. After further washing in medium, the coverslips were mounted in *p*-phenylenediamine on a microscope slide and examined under both phase-contrast and epifluorescence microscopy with a Zeiss photomicroscope. Cells were observed under a 500-fold magnification with TRITC as well as FITC filters to determine the number of cells expressing α gene products or β-gal per approximately 10,000 cells examined.

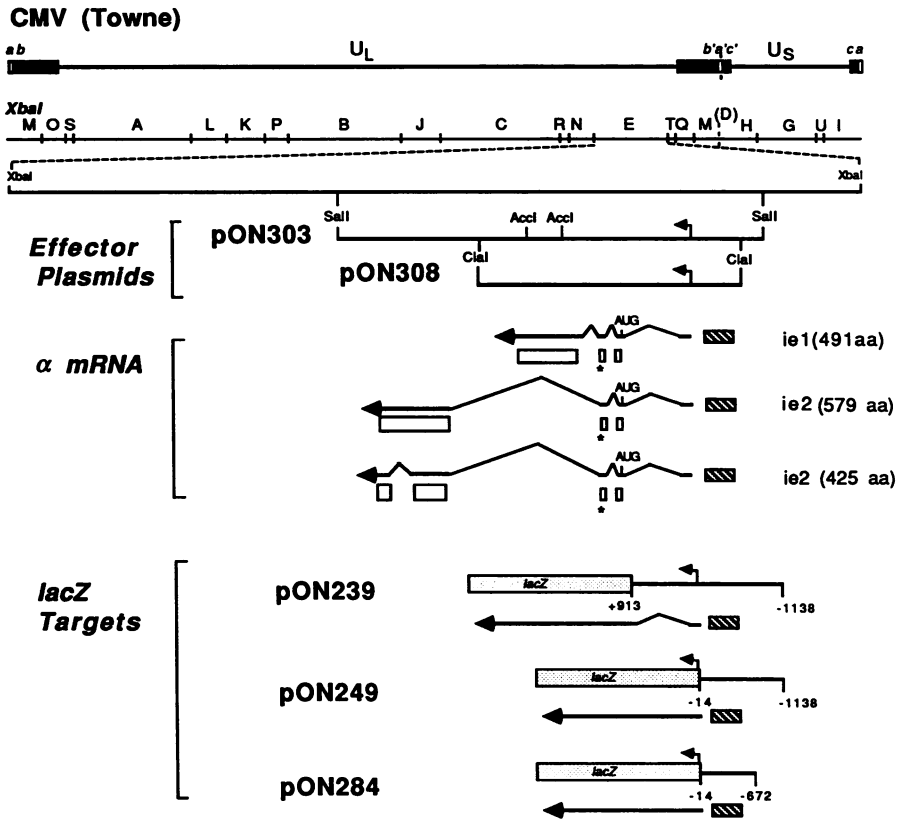

FIG. 1. Organization of the human CMV genome. The 230-kbp human CMV genome is depicted on the top line, with the large boxes indicating inverted repeats (*ab* and *ca*) flanking the unique (U) regions of the L and S components. An *Xba*I restriction map is shown. The *Xba*I E fragment with effector plasmids pON303, carrying the *ie*1 and *ie*2 genes, and pON308 (44), carrying *ie*1, are depicted in the expanded region along with the splicing pattern of the predominant α mRNAs. α protein coding sequences are represented as open boxes, and the epitope for MAb810 is indicated with an asterisk. *lacZ* target constructs pON239 (44), pON249 (14), and pON284 (8) are depicted. The *lacZ* cassette (stippled box) contains a simian virus 40 early polyadenylation site (44). The arrowhead (◄) indicates transcription start sites, and the striped box represents the CMV enhancer on plasmid constructs.

RESULTS

ie2-mediated repression of α gene expression. In previous studies of the effect of α gene products on the α promoter-enhancer, we had observed an activation by *ie*1 products (8, 42). Others (19, 39, 48, 49) reported negative effects of *ie*1 or *ie*2 gene products. The effector plasmids used in these different studies were similar; however, the enhancer-promoter targets varied with respect to indicator gene chosen, chloramphenicol acetyltransferase or *lacZ*, as well as with respect to the point of fusion to the indicator gene. To ascertain the influence of *ie*2 gene products without regard to incidental target gene structure, we examined the autoregulatory influence of this gene directly on α gene expression. We included for comparison in our experiments previously characterized α promoter-enhancer–*lacZ* fusion constructs.

Cotransfection experiments were performed with effector *ie*1 and *ie*2 constructs, and expression of α gene products was monitored by indirect immunofluorescence. Effector plasmids (diagramed in Fig. 1) pON303 (carrying *ie*1 and *ie*2), pON308 (carrying *ie*1), and pON303ΔAcc (carrying *ie*2) each expressed gene products readily detected with murine monoclonal antibody MAb810 (Chemicon) as previously established (27). We observed that the presence of *ie*2 decreased overall expression of α proteins in transient expression assays (Table 1). Figure 2 shows an example of immunofluorescence demonstrating the predominant nuclear localization of ie1 and ie2. All three effector plasmids expressed the epitope reactive with MAb810 (Table 1). Importantly, and consistent with previous reports obtained by using similar plasmids (20, 27, 39), ie2 was expressed from pON303ΔAcc (Table 1). The number of cells expressing α

TABLE 1. Immunofluorescence analysis of gene expression by effector and target constructs

Plasmid(s) transfected	No. of cells[a] expressing:		% cells expressing ie1 and ie2 also expressing β-gal
	α gene products	β-Gal	
Exp 1			
284	0	8	0
284 + 308 (*ie*1)	423	78	19
284 + 303 (*ie*1 + *ie*2)	177	73	41
284 + 303ΔAcc (*ie*2)	30	5	17
Exp 2			
284	0	4	0
284 + 308	133	39	29
284 + 303ΔAcc + 308	39	23	59
284 + 303ΔAcc	13	4	30

[a] Per approximately 10,000 HF cells scanned at 72 h posttransfection.

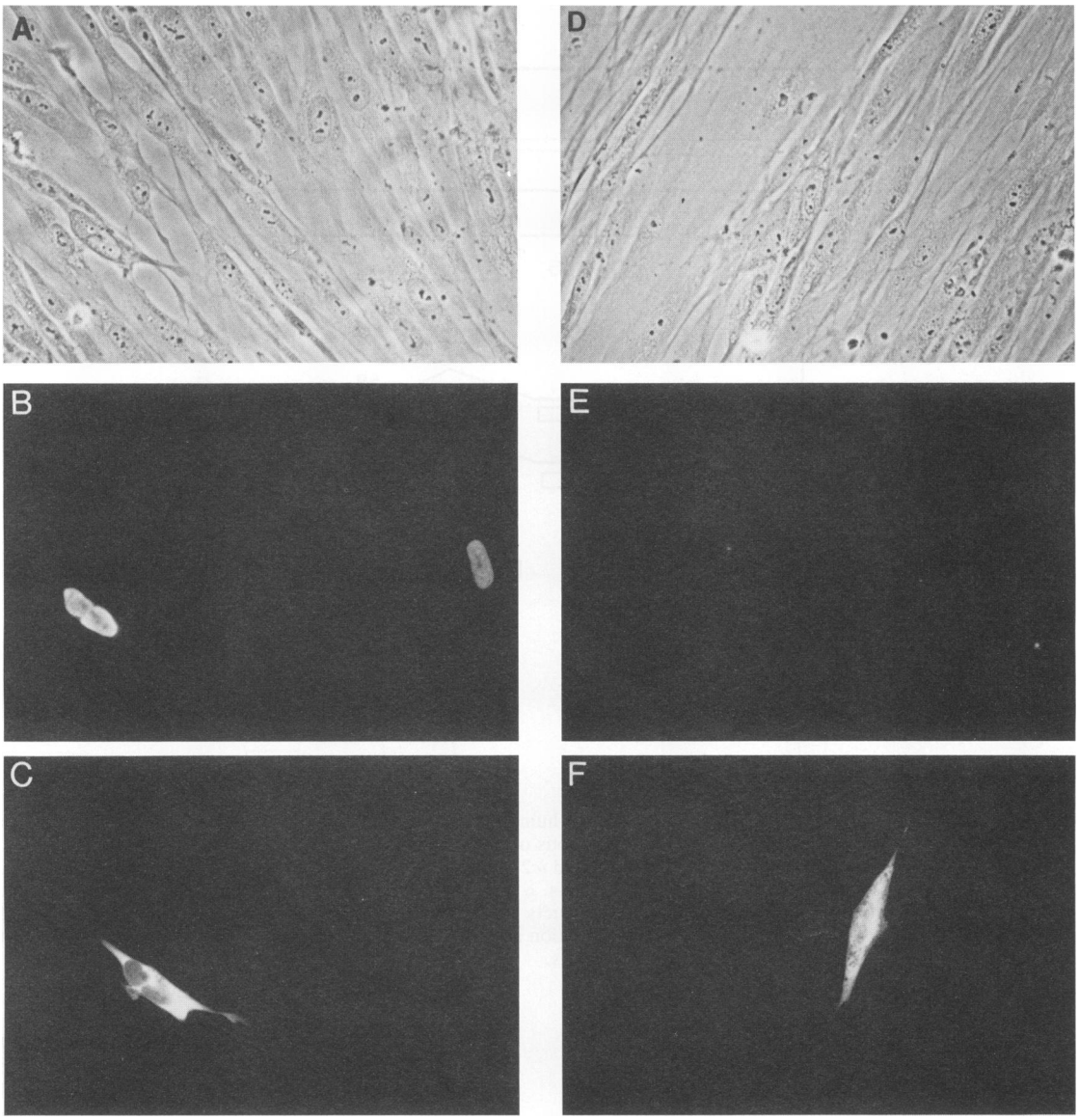

FIG. 2. Double-label immunofluorescence staining on HF cells 72 h posttransfection with either pON284 and pON308 (A to C) or pON284 alone (D to F). (A and D) Phase-contrast photomicrographs showing HF cell monolayers; (B and E) staining with MAb810 and goat $F(ab')_2$ fragments directed against mouse IgG conjugated with TRITC; (C and F) staining with rabbit anti-β-gal antibody and goat IgG anti-rabbit IgG conjugated with FITC. Magnification, ×500.

gene products from pON303 was dramatically reduced compared with pON308, suggesting that the presence of *ie*2 products had a negative effect on expression. When *ie*2 was introduced in *trans* by using pON303ΔAcc, expression from pON308 was also lower than in cells receiving pON308 alone (Table 1). This finding is consistent with reports of others (19, 39, 48) using indicator gene constructs as targets and, in addition, demonstrates that the presence of *ie*2 products clearly had a negative effect on the expression of the intact α gene cluster. This observation supports a role for repression in the biology of CMV.

Because this result appeared to contrast with our previous findings demonstrating that plasmid constructs carrying *ie*1 plus *ie*2 or *ie*1 alone were equally capable of transactivating the α promoter-enhancer (8), we included a *lacZ* target construct in the assay. Inclusion of this indicator construct did not influence the level of expression of α gene products from any of the effector plasmids or the negative effect of *ie*2 on the effector plasmids (unpublished data). Each of these constructs was cotransfected with target pON284 (carrying the *lacZ* gene under control of a 658-bp α promoter-enhancer, from −672 to −14 relative to the transcriptional start site [8; Fig. 1]). Double-label indirect immunofluorescence was performed with MAb810 to detect ie1 and ie2 expression and with a rabbit antibody to detect β-gal expression. Figure 2 illustrates the pattern of expression observed following immunofluorescence staining on an HF culture 72 h after cotransfection with pON284 and pON308. Figure 2B shows two cells expressing *ie*1 and *ie*2 gene products, and one of these cells also showed expression of β-gal (Fig. 2C), indicating transactivation of the α promoter-enhancer by ie1. A small number of cells transfected with pON284 alone ex-

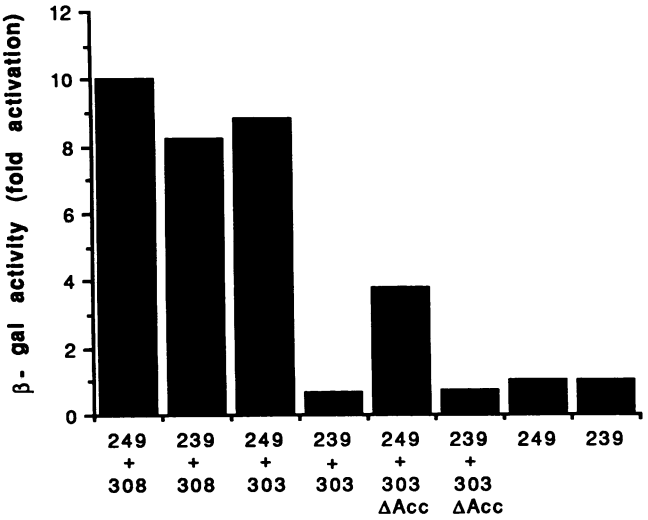

FIG. 3. Repression of expression from the α promoter-enhancer by *ie*2 gene products. The target construct pON249 or pON239 (10 μg) was cotransfected with 20 μg of effector construct pON308 (carrying *ie*1), pON303 (carrying *ie*1 and *ie*2) or pON303ΔAcc (carrying *ie*2) into 3×10^6 HF cells. β-Gal activity was measured at 48 and 72 h after cotransfection on duplicate dishes (8). β-Gal levels are expressed as fold activation measured after cotransfection with an effector clone compared with transfection of pON249 or pON239 alone; the values represent averages of β-gal activities measured from 12 separate transfection experiments. The β-gal activities ranged no more than 10% above or below the indicated values.

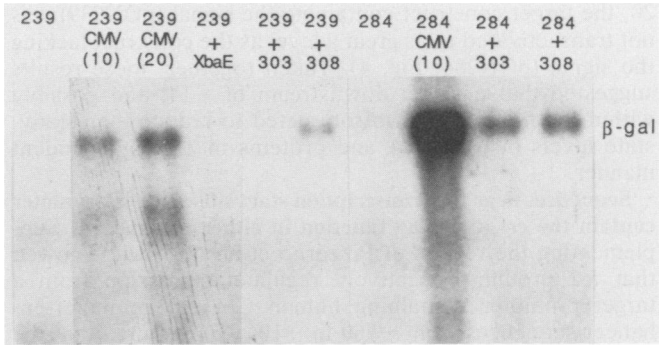

FIG. 4. Blot hybridization analysis to determine steady-state RNA levels in transfected cells. Target plasmids pON239 and pON284 were individually transfected into HF cells (lanes 1 and 7), transfected into cells that were subsequently infected with CMV (lanes 2, 3, and 8), or cotransfected together with effector plasmid pXbaE (lane 4), pON303 (lanes 5 and 9), or pON308 (lanes 6 and 10). Transfections were performed by using 10 μg of target plasmid and 20 μg of effector plasmid. CMV infections were performed by using a multiplicity of infection of 10 (lanes 2 and 9) or 20 (lane 3) PFU per cell. Whole-cell RNA was harvested at 24 h postinfection and 48 h posttransfection; 10 μg of each sample was loaded into a lane on a formaldehyde–1% agarose gel. Following electrophoretic separation and transfer to nitrocellulose, the blot was hybridized with a ^{32}P-labeled RNA probe complementary to the 3′-terminal 199 bp of *lacZ*, which detected a 3.6-kb *lacZ* transcript (indicated by β-gal at the right).

pressed β-gal (Fig. 2F and Table 1) apparently as a result of enhancer activity in the absence of any viral regulatory functions. Consistent with our previous results, immunofluorescence analysis showed that cotransfection of either effector plasmid with pON284 resulted in a similar number of cells in which β-gal expression had been transactivated (Table 1). We interpret this finding to mean that although α gene expression from the two effector plasmids themselves was decreased by the presence of *ie*2, the number of cells that contained sufficient α gene products to activate β-gal expression remained the same (Table 1). Taken together, these results predicted that a *cis*-acting signal was present on the natural genes carried by effector plasmids but absent from our *lacZ* target constructs. This signal was apparently responsible for ie2-mediated repression of α gene expression. These results also suggested that even though it is subjected to shutoff, pON303 makes sufficient α gene products to transactivate the α promoter-enhancer on the *lacZ* target construct.

The major difference between *lacZ* target constructs (pON284 and pON249) and the natural *ie*1 and *ie*1-*ie*2 constructs (pON308 and pON303) was the fact that the target constructs carried enhancer-promoter sequences only to −14 relative to the transcription start site. To determine whether sequences downstream of this site were important in ie2-mediated repression of gene expression, we cotransfected *ie*1 and *ie*1-*ie*2 effector plasmids with a *lacZ* target construct pON239 (44) containing an α gene region from −1138 upstream of the enhancer to +913 bp (i.e., through the first exon and first intron). In transient assays, pON239 was compared with pON249 or pON284, which included upstream sequences extending only to −14. We previously showed that ie1 alone or ie1 and ie2 together transactivated pON284 or pON249 to similar levels (8) and thus have used these two target plasmids interchangeably in our investigation of the regulation of the α locus. In agreement with our previous results, ie1 expressed from pON308 transactivated both pON239 and pON249 to equivalent levels and pON249 was highly transactivated by ie1 and ie2 expressed from pON303 (Fig. 3). pON249 was also transactivated by pON303ΔAcc, suggesting that ie2 can activate expression from the CMV enhancer when the repression signal is absent. In contrast, ie1 and ie2 expressed from pON303 (and ie2 expressed from pON303ΔAcc) failed to transactivate pON239, suggesting that *ie*2 products repressed β-gal expression from pON239 in a manner similar to what was observed with the full-length *ie*1 and *ie*2 constructs. Indeed, levels of expression from pON239 in the presence of ie2 (pON303 or pON303ΔAcc) were consistently below those of pON239 alone (Fig. 3), suggesting that basal expression by the CMV α promoter-enhancer was also repressed by ie2. Thus, the target sequence for ie2-mediated repression was apparently contained within a 927-bp region from −14 to +913 relative to the transcription start site.

To investigate whether this difference in β-gal activity in these cotransfections was a result of a difference in transcriptional levels, an RNA blot analysis was conducted (Fig. 4). Steady-state levels of *lacZ* RNA from either target construct containing (pON239) or lacking (pON284) the target signal were similar when transactivated by pON308, as shown previously (8); however, RNA levels were significantly lower in cells cotransfected with the *ie*1-*ie*2 effector pON303 plus pON239 than in to those cotransfected with pON303 plus pON284. Furthermore, when cotransfection was performed with pXbaE, which carries *ie*1 and *ie*2 plus additional flanking sequences not present in pON303, pON239 expression was repressed (Fig. 4). Finally, when cells transfected with pON284 or pON239 were superinfected with CMV at a multiplicity of infection of 10 or even

20, the target construct containing the signal (pON239) was not transactivated to as great a level as the construct lacking the signal (pON284; Fig. 4). Taken together, these results suggested that a signal downstream of −14, and possibly within the first exon or intron, acted to reduce the steady-state levels of α mRNA and proteins in an ie2-dependent manner.

Sequences near the transcription start site of the α promoter contain the *crs* and may function in either orientation. Supplementing the results of Pizzorno et al. (39), who showed that *ie2* products negatively regulate expression from a target promoter containing human CMV α promoter-enhancer sequences from −760 to +10, we predicted that the *crs* should be contained within the sequences between −14 and +10. To determine whether this sequence alone was sufficient for repression, a double-stranded oligonucleotide representing −14 through +9 was synthesized, inserted into target indicator plasmids, and tested in transient cotransfection assays for the ability to confer ie2-mediated repression. This oligonucleotide was cloned in both orientations (pON249crs and pON249crs′; Fig. 5) into a position such that the expected transcription initiation site in the sense construct (pON249crs) would be approximately 12 bp upstream of its normal position (14, 59). Following cotransfection with pON303, only constructs containing this oligonucleotide were repressed, thereby showing that the *crs* was contained within this −14 to +9 region (Fig. 5). Effector pON303, expressing ie1 and ie2, but not effector pON308, repressed β-gal expression. Again, as for the previously tested target constructs, basal expression of these target constructs was repressed in the presence of the *ie2* construct pON303ΔAcc (unpublished data). Given the demonstration that this sequence functions as a repression signal independent of orientation, we believe that repression is being mediated at the level of transcription.

Oligonucleotides containing substitution mutations throughout the −14 to +9 region were synthesized and cloned into the *Hin*dIII site of pON249, maintaining the spacing as in pON249crs (Fig. 6). These target constructs were then cotransfected with pON303, and β-gal activity was measured 48 h later. Sequences within the 5′ portion of the signal were crucial for repression by ie2 (pON249*crs*A, pON249*crs*B, and, to a lesser extent, pON249*crs*C were no longer repressed by pON303); however, a mutation that disrupted the extreme 3′ end of the −14 to +9 oligonucleotide (pON249*crs*D) was not repressed by pON303 at all (Fig. 6). An oligonucleotide representing the sequences from −14 to +1 (pON249*crs*E) was repressed to a level equivalent to that measured from pON239 or pON249crs. In all cases of the mutant *crs* constructs, the *crs* was cloned in both orientations and subsequently assessed for function in transient expression assays; repression was consistently found to be independent of the orientation of the signal (unpublished data).

In the pON249crs substitution mutation series, the transcription start site of the α promoter would be expected to have shifted approximately 12 nucleotides upstream of its normal position. To ensure that the −14 to +1 signal would function to mediate repression in its precise natural location within the α promoter, pON239*crs*E was constructed. This plasmid was similar to pON239 except that it contained a deletion of nucleotides +2 to +913, thus removing the first exon and first intron of the α gene locus. pON239*crs*E responded exactly as pON239 when cotransfected into cells with pON303: β-gal expression was repressed from both targets (unpublished data). Furthermore, β-gal expression

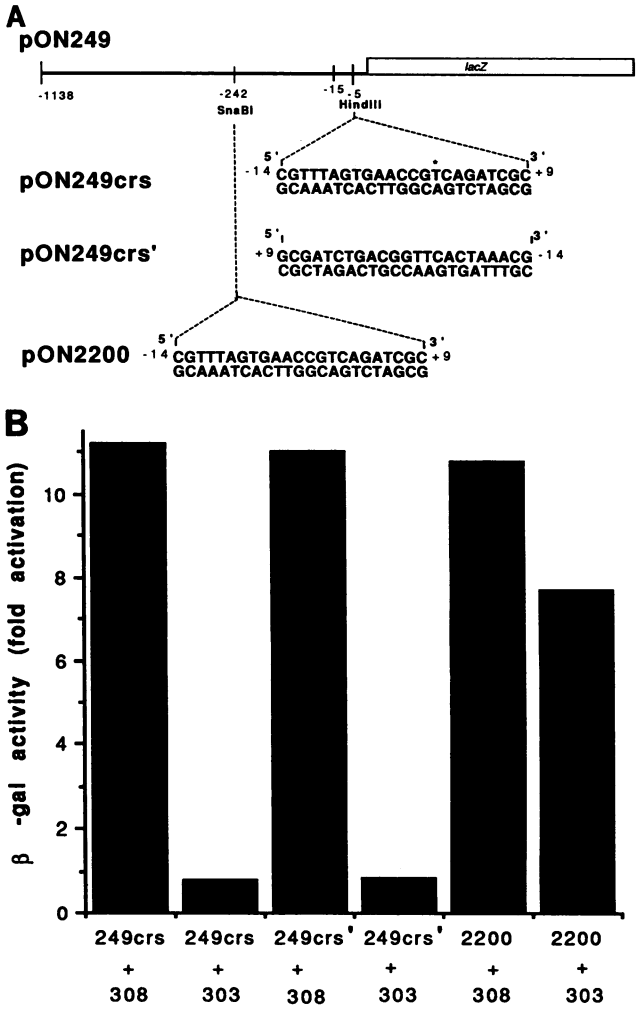

FIG. 5. Occurrence of ie2-mediated repression via a region −14 to +9 spanning the transcription start site. (A) *lacZ* target constructs. pON249 contains enhancer sequences from −1138 to −14 fused to *lacZ* (14). pON249crs contains the same upstream sequences as pON249 plus an insertion of the double-stranded *crs* oligonucleotide in positive orientation (5′ −14 to +9 3′) at the unique *Hin*dIII site at −5 (relative to the transcription start site). pON249crs′ contains the *crs* oligonucleotide cloned in opposite orientation (5′ +9 to −14 3′) into the same *Hin*dIII site. pON2200 shares enhancer sequences with pON249 but contains the *crs* oligonucleotide cloned (in positive orientation) into the *Sna*BI site at −242. The asterisk depicts the transcriptional start site (+1) for the *crs* (−14 to +9) sequence when present in its natural setting within the viral genome (59). (B) Repression of *crs* when positioned near the transcription start site. The target constructs containing the *crs* in sense (pON249crs) or antisense (pON249crs′) orientation near the transcription start site or upstream in the enhancer (pON2200) were cotransfected with pON303 or pON308 as indicated. β-Gal activity was assayed as described for Fig. 3.

from pON239*crs*E was repressed in an ie2-dependent manner just as it had been from target pON249*crs*E. These data confirmed that the sequence −14 to +1 of the α promoter was functional as a *cis* repression element when placed in its natural position within the α promoter-enhancer and that the results obtained from the pON249crs substitution mutation series accurately reflected the natural setting. Notably, this sequence is not repeated elsewhere in the CMV genome; the

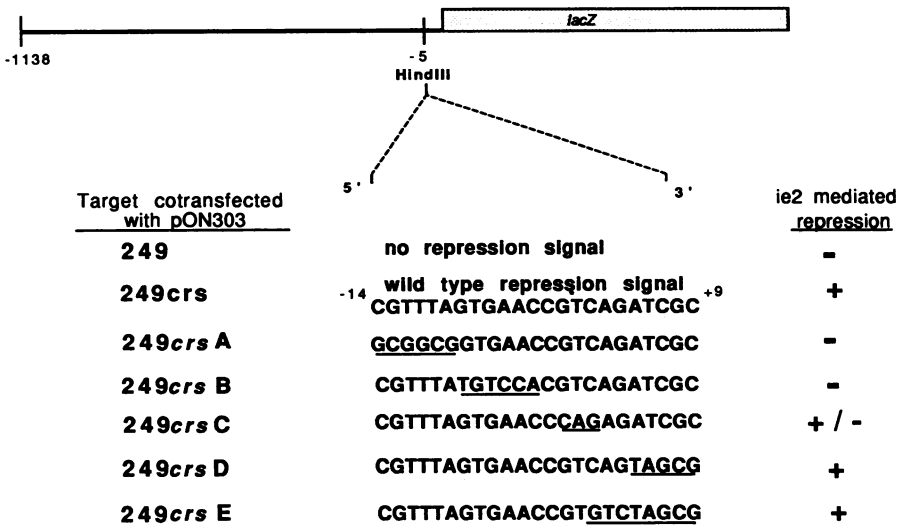

FIG. 6. Location of the *crs* for ie2-mediated repression upstream (between −14 to +1) of the natural transcription start site. Substitution mutations within the *crs* oligonucleotide were synthesized and cloned into the *Hin*dIII site of pON249, shown as constructs 249*crs*A through 249*crs*E. Nucleotides within the −14 to +9 region that have been substituted are underlined. These mutant constructs (10 μg) were cotransfected with pON303 (20 μg) into 3×10^6 HF cells and assayed for β-gal activity 48 and 72 h posttransfection as described for Fig. 3. Results were identical when 4×10^5 cells were transfected with either 2 or 4 μg of effector plasmid and 1 μg of target plasmid. Similar results were obtained from transfection of Vero cells (data not shown).

closest homologies require three mismatched base pairs and are not located near known α genes (unpublished data).

The *crs* does not function when positioned far from the start site of transcription; complex enhancer sequences are not required to mediate repression. To determine whether the *crs* needed to be positioned near the transcription start site to function, we cloned the signal at −242 in pON249 (pON2200). Following transfection, pON2200 was not repressed by *ie*2 (Fig. 5), suggesting that proximity to the promoter may be critical for *crs* function.

There were at least two possibilities for the observed results: either the *crs* must be present very close to the transcription start site or the signal requires additional enhancer sequences to be functional. To distinguish between these two possibilities, we first constructed the pON249crs enhancer deletion series. These constructs (pON283crs, pON2044crs, and pON2046crs) carry deletions in upstream enhancer sequences and contain the −14 to +9 signal near the transcriptional start site of the CMV α promoter at the same unique *Hin*dIII site used to construct pON249crs (8). Parental *lacZ* constructs (pON249, pON283, pON2044, and pON2046) do not carry the repression signal. Following cotransfection with pON303, all constructs carrying the *crs* were repressed in an ie2-dependent manner even though the enhancer repeat motifs and consensus binding sites for a variety of cellular transcription factors were successively deleted (Table 2). Therefore, the *crs* was able to function on a 75-bp α promoter containing a TATA box, an Sp1 site, a CAAT box, and one 19-bp repeat element with its associated ATF binding site. Repression by ie2 via the *crs* was not dependent on the highly complex enhancer.

ie2-mediated repression of a heterologous promoter. Given the requirement that the *crs* be placed near the transcription start site to function in ie2-mediated repression, the signal may require other elements surrounding this region. To determine whether the *crs* could confer ie2-mediated repression when present near the start site of a promoter other than the CMV α promoter, we inserted the this signal near the transcription start site of the HSV-1 TK promoter. As expected from the HSV literature (12, 15, 38), the HSV TK promoter without the *crs* (pON2201) was very well activated by a plasmid (pRB201) carrying the *Hin*dIII HM junction fragment with the HSV α0, α4, and α27 genes (Fig. 7). Addition of pON303 did not alter the ability of the HSV α proteins to transactivate the TK promoter and had only a small positive effect on expression from pON2201 in the absence of pRB201. When the *crs* was used to replace sequences near the transcription start site of the HSV-1 TK promoter (pON2202), transactivation by HSV-1 α proteins was not affected (Fig. 7). To determine whether ie2 was able to repress expression from pON2202, we conducted transfection experiments with pRB201, pON2202, and pON303. pON303 repressed HSV-1 α protein-activated expression of pON2202 (Fig. 7). Furthermore, pON303ΔAcc, which expressed functional *ie*2 gene products, was able to replace pON303 in this assay and mediate repression (unpublished data). In the absence of pRB201, pON303 had no effect on expression from pON2202. Thus, ie2-mediated repression can be conferred on a heterologous promoter by *crs* if the promoter is transcriptionally active.

TABLE 2. Results showing that delections removing enhancer elements are still subject to ie2-mediated repression via *crs*

Target plasmid	No. of:				Fold activation upon cotransfection with 303
	21-bp repeats	19-bp repeats	18-bp repeats	16-bp repeats	
249	2	4	5	3	10
249crs	2	4	5	3	0.8
283	0	2	2	1	5.4
283crs	0	2	2	1	0.8
2044	0	1	2/3	0	3.2
2044crs	0	1	2/3	0	0.8
2046	0	1	0	0	2.8
2046crs	0	1	0	0	0.9

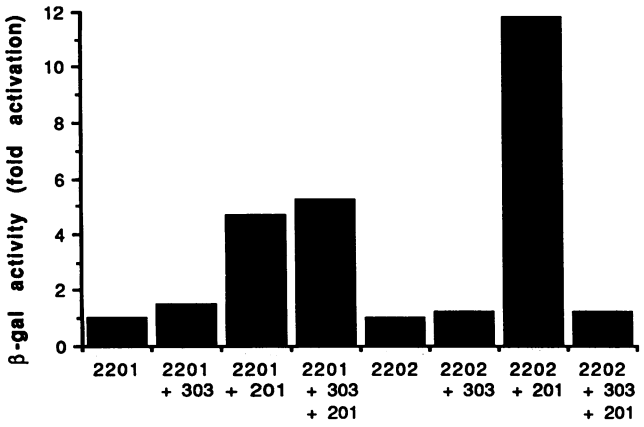

FIG. 7. Demonstration that the −14 to +9 *crs* confers ie2-mediated repression upon a heterologous promoter. A 10-μg sample of target constructs pON2201 (an HSV-1 TK promoter–*lacZ* fusion) or pON2202 (a TK promoter–*lacZ* fusion with the *crs* oligonucleotide inserted at −16 relative to the transcription start site) was cotransfected with 20 μg of effector construct pON303 (carrying CMV *ie*1 and *ie*2) alone, pRB201 (carrying HSV-1 α0, α4, and α27 genes) alone, or both pON303 and pRB201 together. β-gal activity was assayed as described for Fig. 3.

DISCUSSION

We have demonstrated that CMV *ie*2 proteins negatively regulate the α promoter-enhancer through a *cis* signal, *crs*, contained in the sequence 5′-CGTTTAGTGAACCGT-3′, which naturally overlaps the transcriptional start site (between −14 and +1) of the CMV α promoter-enhancer. Our primary interest has been the ability of ie2 to inhibit activation of the α gene by ie1; however our data indicate that the gene expression is repressed even in the absence of enhancer transactivation. The signal may be moved to a heterologous promoter and confer ie2-dependent repression on that promoter. Steady-state RNA levels from *crs*-containing promoters are reduced in the presence of ie2, and the *crs* is functional in either orientation when placed near but upstream of the transcription start site, features that strongly suggest that repression occurs at the transcriptional level.

While a great deal has been learned over the past several years concerning mechanisms of activation of eucaryotic gene expression, little is known about negative regulation, or repression, of transcription. Although the list of eucaryotic repressors is quite short, a number of mechanisms for their function have been proposed (28). Our current concept of repression of eucaryotic gene expression is largely derived from work conducted in procaryotic systems where the overriding theme demonstrates a repressor protein binding to DNA at or near the transcriptional start site such that the interaction of general transcription machinery, including RNA polymerase, with the promoter is blocked (24). Examples of direct repression of gene expression via target signal have been presented for bovine and human papillomaviruses, in which the E2 protein can mediate shutoff by binding to sites near a transcription start site (41, 52). Direct binding to DNA was initially believed to be responsible for simian virus 40 T-antigen repression of early gene expression; however, recently indirect mechanisms have been proposed (18, 34). During the regulation of several drosophila homeobox proteins, a related type of direct repression occurs through competition for the same or a similar DNA-binding site upstream of a promoter that relies on activator binding and activity for expression (22); repressor binding prevents activator binding, resulting in repression.

Our results suggest that ie2-mediated repression occurs directly or via an induced cellular protein, by blocking access of RNA polymerase to the promoter or somehow interfering with assembly or processivity of the transcription complex. The *ie*2 proteins have not as yet been shown to possess any sequence-specific DNA-binding activity or to contain regions of significant homology to other transcriptional regulators; therefore, it is possible that ie2-mediated repression involves an interaction with a cellular protein. Clues to the function of ie2 in repression may come from its well-established role as a heterologous transactivator (58) similar to adenovirus E1A (3). In the absence of the *crs*, ie2 functions as a transcriptional activator of a variety of promoters, including the α promoter itself (6, 8, 9, 11, 12, 20, 25, 39, 46, 58; this work). A complete analysis of *cis* targets responsive to ie2 transactivation has not been completed; however, it appears that ie2 may be mediating transactivation indirectly via a range of cellular transcription factors along the same lines as adenovirus E1A. Although cellular transcription factors appear to be activated by viral proteins like E1A and ie2, possibly certain cellular factors induced by ie2 act to downregulate transcription through the *crs*.

In light of the above observations, it is interesting that ie2 acts as a dominant transcriptional repressor even in the presence of the activator protein ie1 as long as the *crs* is present at the target promoter. This dominant repression by ie2 appears to extend to other heterologous systems such as the TK gene activated by HSV α gene products. This finding suggests that ie2 may exert its repression function via one of at least two mechanisms depending on the concentration or availability of other viral proteins and cellular factors with which it may interact. One possible mechanism would have ie2 itself or an ie2-induced cellular protein binding to the *crs* and preventing transcription initiation. The data presented here suggest that ie2-mediated repression occurs in a relatively straightforward way, by preventing transcription through direct or indirect occupation of the *crs*, a mechanism reminiscent of classical procaryotic repression. Whether ie2-mediated repression occurs by steric hindrance as has been demonstrated for a number of procaryotic repressors, including *Escherichia coli lac* (2), P22 *mnt* and P22 *arc* (64), and λ *cro* and λ cI (23, 24), or by affecting the function of bound transcriptional machinery remains an open question. Second, ie2 itself or an ie2-induced protein may repress transcription by blocking some component of the transcription apparatus which is particularly sensitive to the presence of *crs* between the TATA box and the transcriptional start site. A similar type of mechanism has been proposed to explain how the yeast *GAL1* gene is repressed by negative regulatory factors bound between the upstream activator sequence and the TATA region (5, 55) and for the function of bovine papillomavirus E2 (41).

The CMV *ie*2 proteins possess a variety of functions: they transactivate homologous and heterologous promoters and repress their own expression as well as the expression of ie1. Indeed, in the context of the intact virus, transcription of *ie*1 and *ie*2 peaks early in infection and decreases thereafter, consistent with repression occurring at this locus (47, 49, 51). The multiple functions of the *ie*2 proteins are likely to be critical elements of both productive and latent infections. Furthermore, their diverse functions will probably involve interactions with accessory cellular proteins, and further

work should determine the role of these factors in ie2-mediated repression and activation of α gene expression.

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Exhibit 4

Molecular Genetic Analysis of Cytomegalovirus Gene Regulation in Growth, Persistence and Latency

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1 Introduction

For the great number of RNA and DNA viruses that persist in the host following primary infection, two common tenets emerge: viral gene expression is generally downregulated or altered during persistent infection, and the host immune response fails to detect and clear virus-infected cells (OLDSTONE 1989). While evasion of immune surveillance is important in all persistence, viral gene products may play a direct role as regulatory functions in certain persistent viruses. Herpesvirus latency is associated with a dramatic restriction of viral replication and gene expression, suggesting that these viruses encode gene products that downregulate replication functions in certain target tissues (STEVENS 1980; JORDAN 1983; ROIZMAN and SEARS 1987; BAICHWAL and SUGDEN 1988). The best candidates for latency-regulatory genes are in the α (immediate early) class (LEIB et al. 1989) as well as genes that are expressed during latent infection (STROOP et al. 1984; CROEN et al. 1987; STEVENS et al. 1987; ROCK et al. 1987; BAICHWAL and SUGDEN, 1988).

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1.1 General Biology

Human cytomegalovirus (CMV, human herpesvirus five) is a ubiquitous human pathogen causing a broad range of clinical illness primarily in very young or immunocompromised hosts and is the prototype member of a larger group of biologically related herpesviruses, the β-herpesviruses (Stinski 1983; Spector and Spector 1984; Alford and Britt 1985; Griffiths and Grundy 1987). Representatives of the β-herpesvirus subfamily, all cytomegaloviruses, are found throughout nature in many animal species (Plummer 1973). As is characteristic of all herpesviruses, CMV persists in the host following primary infection and remains latently associated with infected individuals for life (Jordan 1983; Ho 1982). The precise sites and mechanism of CMV latency remain unclear, although latent virus appears to have a broad tissue distribution as indicated by the transmission of CMV following transplantation of different organs. Salivary gland, blood cells, spleen, and kidney are suspected tissue targets. Very little is known about the molecular events or viral functions that control persistence and latency. Given the extreme difficulty in studying these processes with human CMV in humans, cell culture models of human CMV persistence and animal studies (particularly with murine and guinea pig CMV) have been pursued with the expectation that they might provide insights into the human CMV-host interactions.

In humans as well as in animal species, the respective CMV silently infects an overwhelming majority of the population before adulthood but causes little or no overt illness in most individuals (Ho 1982; Alford and Britt 1985). Each of the different CMVs replicates in salivary gland, and, because of persistent and recurrent replication in this organ, oral secretions seem to be a primary source of high-titered virus for dissemination within the population. In humans and other species the virus causes only mild symptoms during primary infection and remains an inocuous passenger in the immunocompetent individual. Human CMV reveals its pathogenic capabilities in the developing fetus, in the newborn, or in immunocompromised individuals. Even though the virus can spread efficiently during primary infection, persistent and latent virus appears to be a major contributor to CMV disease. A latently infected mother may reactivate virus and pass it to her fetus. Blood transfusion, from latently infected seropositive donors, is a major source of CMV transmission in hospitalized patients. Latent virus (either within an individual or introduced along with a transplant) is usually the infectious source in CMV-related illness during immunosuppression or immunodeficiency. Thus, the molecular basis of CMV persistence and latency is of central importance to our understanding of CMV pathogenesis.

1.2 Host Range and Similarity to Other Herpesviruses

CMV is highly restricted in its host range in cell culture. Only differentiated cells appear to be permissive; undifferentiated or transformed cell lines are nonpermissive

Exhibit 5

CURRICULUM VITAE

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Education and Employment Record (current appointments in bold):

Rutgers University, New Brunswick, New Jersey	A.B. Microbiology	1970-74
University of Iowa, Iowa City, Iowa	Ph.D. Microbiology	1974-79
USPHS Predoctoral Trainee in Cellular and Molecular Biology		1975-78
The University of Chicago, Chicago, Illinois	Postdoctoral	1979-83
USPHS Postdoctoral Trainee in Virology		1979-81
(University of Bologna, Visiting Scientist, 1980)		
Leukemia Society of America Special Fellow		1981-83
Stanford University School of Medicine, Stanford, California		
Assistant Professor of Microbiology & Immunology		1983-89
Associate Professor of Microbiology & Immunology		1989-95
Chairman of the Department of Microbiology & Immunology		1995-99
Professor of Microbiology & Immunology		1995-06
Professor Emeritus		5/2006
Stanford University, Stanford, California		
Associate Dean of Research		2000-01
Emory University, Atlanta Georgia		
Robert W. Woodruff Professor of Microbiology & Immunology		2006-2021
Emory Vaccine Center		2006-2021
Professor Emeritus		1/2022

Professional leave:	SyStemix, Palo Alto, California	1990 (6 mo.)
	Aviron, Mountain View, California	1995 (6 mo.)
	MedImmune-AstraZeneca Distinguished Fellow	11/2008-1/2011

Honors and Awards:

Closing Keynote - 44th International Herpesvirus Workshop (2019)
Gertrude and Werner Henle Lectureship, University of Pennsylvania (2018)
NIH Director's Transformative Award (funded by NIAID 2015-2019)
Nirit and Michael Shaoul Fellow (two week visiting professor), Tel Aviv (2015)
Inaugural Weller-Smith Oration, CMV/Betaherpesvirus Workshop, Brisbane (2015)
Keynote – Second International Herpesvirus Forum, Guangzhou (2014)
Herpes Liason Award – Virginia Regional Herpes Symposium (2014)
Emory 1% Award (2012)
Fellow of the American Academy of Microbiology (2012)
Keynote Speaker – International Herpesvirus Workshop, Gdansk (2011)
Keynote Speaker - Nebraska Center for Virology 10th Anniversary (2010)
Distinguished Fellow, MedImmune (2008 - 2011)
Belleville Wall of Recognition (2008)
Maurice Hilleman Lecturer, The University of Chicago (2008)
Georgia Cancer Coalition Eminent Scholar (2006-2011)
Robert W. Woodruff Chair Professorship (2006-present)
Louisiana State University Visiting Faculty (2004)
Stanford University Fellow (2002-2004)
Pfizer Visiting Professor in Infectious Diseases, University of Oklahoma (2001)
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SmithKline Beecham Fellow (1995-96)
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National Institutes of Health Wallace Rowe Lecture (1993)
American Cancer Society Faculty Research Grant (1984-1993)
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National/Foreign Review Panel Memberships (current membership in bold):

NIH Reviewers Reserve (ad hoc) reviewer (1994-pres)
UK MRC ad hoc reviewer (2010-pres)
Welcome Foundation ad hoc reviewer (2009-pres)
ME/CFS Collaborative Research Centers, reissued (2023)
ME/CFS) Collaborative Research Centers (2022)
NIAID DMID BAA Development of Medical Countermeasures for Biothreat Agents, Antimicrobial-Resistant Infections and Emerging Infectious Diseases (2021)
NIH Vaccines Against Microbial Disease Study Section (2014-2015) ad hoc reviewer.
NIH Special Emphasis Review Study Section RFA "Omics Technologies for Predictive Modeling of Infectious Diseases" (2013)
NIH Special Emphasis Review Study Section BAA '"Targeted Clinical Research to Address Select Viral Infections" (2011)

Topics in Virology Study Section (*ad hoc* 2007-2009)
UK MRC Virology Focus Strategy Review Group (2006-2008)
Institute of Medicine (National Academy of Sciences) Consultant, Committee on Review of Priorities in the National Vaccine Plan, 2008
NIDOCD Review Panel on CMV-related Hearing Loss (2004)
NIH Virology B Study Section (*ad hoc* 2004)
Advisory Panel to Office of AIDS Res on Opportunistic Infections (1995-96)
NIH-NIAID Spec Review: Molec. & Struc. Appr. Antiviral Drug Design (1994)
NIH Experimental Virology Study Section (1990-1994)
NIH-NIAID Special Review - Animal Models of Human Viral Infections (1990)
NIH-NIAID Workshop on Opportunistic Infections in AIDS (1989)
NIH Small Business Administration Study Section (1988)
USDA Biotechnology Study Section (1986-88)

Editorial Board:
***Journal of Virology* (1991-present), *Virology* (1991-present)**, ***PeerJ* (2012-present), *PLoS Pathogens* (2014-present), *eLife* (2016-present), *J. Biol. Chem.* (2018 – present)**
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Panel Member: Wellcome Foundation (2009), United Kingdom MRC reviewer (1999, 2003-2008), NIH Clinical Sciences I Study Section (1985), NIH Site Visit Panels (1986, 1987), NIH Virology Study Section (1988), NIH-NIAID Microbiology and Infectious Disease Research Committee (1989), USDA-Hatch Grant Program Reviewer - University of Nevada (1989), NIH-NIAID Board of Scientific Counselors (1989), USDA Biotechnology Study Section (1989-95).

Reviewer: the USDA Biotechnology Study Section (1989-95) and the NIH-NIAID Board of Scientific Counselors (1989). I have also served as a reviewer or panel member for the Medical Research Council (United Kingdom), the Wellcome Foundation, the Australian Medical Research Council, the Canadian National Institute of Health, the Israel Science Foundation, the Veterans Administration, the National Foundation March of Dimes, the National Science Foundation and the Natural Sciences and Engineering Research Council of Canada.

Invited Reviewer:
Journals: *Science, Nature, Cell, Immunity, Nature Immunology, Nature Medicine, Molecular Cell, Cell Host Microbe, Journal of General Virology, Virus Research, Intervirology, Archives of Virology, Journal of Viral Immunology, Proceedings of the National Academy of Sciences, Journal of Clinical Microbiology, Journal of Infectious Diseases, New England Journal of Medicine, Journal of Experimental Medicine, Blood, Journal of Immunology, Journal of Interferon and Cytokine Research, PLoS Pathogens, J. Biol. Chem., Viruses, Frontiers*

Consultant/Advisory Board Member:

Current Academic and Nonprofit Programs*:*

National Institutes of Health, NIAID, Dale and Betty Bumpers Vaccine Research Center Scientific Advisory Working Group (2017 – present)

Access to Advanced Health Institute (AAHI) Board of Directors (2022 – present), Corey Casper, CEO (formerly known as Infectious Disease Research Institute)

Current Companies:

Scientific Advisory Boards:

ViroThera, Cambridge UK (2020 – present)
Omios Pharma, Alameda CA (2021 – present)
Blue Lake Biotechnology, Los Gatos CA (2022 – present)

Consultant:

Stamford Pharmaceuticals, Austin TX (2023-present; formerly Ascend Biopharma (2016)
CoCrystal Pharma, Seattle WA (2007 – 2016; 2021 – present)
GlaxoSmithKline, London UK (2022 – present)
Oxymo Technologies, Paris, France (2023 – present)
Asha Therapeutics, Tampa FL (2024 – present)
AstraZeneca, London UK (2024 – present)

Past scientific advisory board member *(for-profit)**:***
Agenovir, Inc. (2015 – 2018; acquired by Vir Biotechnology in 2018); ChemoCentryx, Inc. (1997 – 2018; acquired by Amgen in 2022); CoCrystal Pharma, Inc (2007 – 2016); GlobeImmune, Inc (2003 – 2015); Immune Design, Inc. (2012 – 2014; acquired by Merck in 2019); Ribozyme Pharmaceuticals, Inc. (RPI, 1992-2001; renamed SIRNA, acquired by Merck in 2007); Aviron (1992-2002; acquired by MedImmune, then AstraZeneca); Chiron Corporation (1991-92); Schering-Plough (1984-1994); Syntro, Inc. (1985-88)

Past scientific advisory board member *(nonprofit)**:***
International AIDS Vaccine Initiative Scientific Advisory Committee (2014 - 2017); Louisiana Biomedical Research Network (2004 -2017); Nebraska Center for Virology (COBRE) (2000 - 2015); HudsonAlpha Institute for Biotechnology R10K (2011-2012);

Previous or occasional consulting *(for-profit and nonprofit)**:***
US Department of Justice (2022-2023); Krog and Partners (2022); DynaVax (2020 – 2022); DNAtrix (2019); Tendel Therapies (2019); Merck (2014 – 2019);
Foley Hoag/Selendy & Gay (2018 – 2019); Pfizer (2011, 2014); GlaxoSmithKline (2011, 2012, 2014); Paul Hastings (2012 – 2013); MedImmune/AstraZeneca (employee 2008 – 2011; consultant, 2011 – 2012; 2016 – 2018); Stanford Ovarian Cancer SPORE (2012); Boehringer Ingelheim (2011); Western Regional Center of Excellence for Biodefense and Emerging Infectious Diseases Research (2010); Berlex (2005); Genzyme/ILEX (2004-2005); 9th District Court, Judicial Scientific Advisor (2000-2003); ImmunoGen, Inc. (1995-2002); GeneTrol (2001-2002); Parke-Davis (Warner-Lambert) (1998-1999); Glaxo-Wellcome Herpesvirus Consultancy Group (1996-1998); Searle-Monsanto (1994-1995); SyStemix, Inc. (1990)

SPONSORED RESEARCH SUPPORT:

COMPLETED (grants held in the three years before retirement from Emory University)

Project Number: 5 RO1 AI020211-33 (Mocarski, PI) **Dates of Project:** 12/01/16-10/31/21
Source: NIH/NIAID **Annual Direct Costs**: $250,000
Title: Cytomegalovirus DNA replication and inversion.
Major Goals: (1) Study cell death pathways controlling viral dissemination. (2) Investigate cell death pathways controlling latency and persistence. (3) Elaborate the contribution of cell death to innate and adaptive immunity.
Percent Effort: 2.0 calendar months

Project Number: none (Mocarski, PI; Mandal, PL) **Dates of Project:** 03/01/21-02/28/23
Source: Halle Institute for Global Research; Emory-Tel Aviv Collaborative research Grant.
Title: Programmed Cell Death Pathway in Virus-Triggered Lung Inflammation
Major Goals: Investigate whether (i) death; (ii) death-independent functions; and/or (iii) necroptotic extracellular vesicles, contribute to cytokine/chemokine production from lung epithelial cells and fibroblasts.
Percent Effort: 0 calendar months

Project Number: none (McCarty, PI; Mandal, PL) **Dates of Project:** 12/01/20-11/30/21
Source: Emory funds.
Title: Role of Programmed Cell Death Signaling in Inflammatory Intestinal Injury
Major Goals: Investigate the contribution of programmed cell death (PCD) pathways including apoptosis, necroptosis and pyroptosis, as well as inflammasome-dependent inflammatory cytokine activation, to inflammation and inflammatory injury in a murine model of cystic fibrosis.
Percent Effort: 0 calendar months (Dr. Mandal is an instructor in my group)

Project Number: 1 R21 AI142507-01 (Mocarski, PI) **Dates of Project:** 07/25/19-06/30/21
Source: NIH/NIAID **Annual Direct Costs**: $125,000
Title: 3-D Culture Models.
Major Goals: Develop human and mouse lung tissue models to investigate the contribution of cytomegalovirus to airway disease, with a focus on virus-encoded and induced cytokines.
Percent Effort: 1.4 calendar months

Project Number: 2 R01 AI118853-01 (Mocarski, PI) **Dates of Project:** 6/01/15-5/31/19
Source: NIH/NIAID Director's Transformative Award **Annual Direct Costs:** $250,000
Title: Innate activation and death signals in health and disease.
Major Goals: (1) Optimize allogeneic engraftment by manipulating cell death pathways by controlling cell death. (2) Enhance nuclear reprogramming by eliminating detrimental innate cell death. (3) Determine the contribution of apoptosis and necroptosis to inflammatory disease in mouse models.
Percent Effort: 20% (2.4 calendar months)

Past Trainees

Sabbatical: Marie Jo Masse, Ph.D. (1991-92)
Lawrence Corey, M.D. (1994)
Dana Wolfe, M.D. (1998-99)
Maria Paola Landini, M.D. (1999)

Richard R. Spaete, Ph.D. (1983-86)
(retired)
Consultant

(Last position)
Senior Director, MedImmune/AstraZeneca

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M. Faraz Nasseri, Ph.D. (1986-88)
(retired)

(last position) Program Officer, NIH/NIAID

Gavin W. G. Wilkinson, Ph.D. (1987-88)
Professor Emeritus (retired)

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(last position) Institute of Microbiology
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(retired)
(last position) Senior Scientist
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Julie Cherrington, Ph.D. (1987-90; Ph.D. 1990)
Chair of Board
Actym Therapeutics, Inc.
Tolremo Therapeutics

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Venture Partner
Brandon Capital Partners

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Jeffrey Vieira, Ph.D. (1988-93)
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(Last position) Research Scientist
Fred Hutchinson Cancer Res Center

William C. Manning, Ph.D. (1984-1990; Ph.D., 1990)
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(Last position) Vice President, Laboratory Operations SEER
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Richard Greaves, Ph.D. (1991-94) (Last position) Investigator
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Gregory Duke, Ph.D. (1991-94) (Prior position) Senior Director Virology
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Rhonda Cardin, Ph.D. (1989-94) Pfizer, Inc.
Executive Director, Anti-Infectives 401 N Middletown Rd
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Janice Brown, M.D. (1992-94) Divisions of Hematopoietic Cell
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Mark Prichard, Ph.D. (1992-96)
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Yu-Chun Lin, M.D./Ph.D. (1991-97; Ph.D, 1997)
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Gabriele Hahn, M.D. (1994-97)
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Major Invited and Plenary Presentations:

1984 Keystone/UCLA Symposium - Herpesvirus
1985 International Herpesvirus Workshop - Ann Arbor
1986 International Herpesvirus Workshop - Leeds
1987 Animal Cells and Viruses Gordon Conference - Tilton
International Congress of Virology - Edmonton
1988 Transfusion-Associated Infections and Immune Response - San Francisco
Banbury Conference on Virus Vectors - Cold Spring Harbor
The Albany Conference - Viral Vectors – Troy NY
International Herpesvirus Workshop - Irvine
1989 First US-Japan Biotechnology Meeting - St. Petersburg FL
Second International Cytomegalovirus Workshop - San Diego
1990 Pathogenesis of Cytomegalovirus-Associated Diseases - Irvine
Annual Meeting of German Virologists - Ulm
International Congress of Virology - Berlin
International Herpesvirus Workshop - Georgetown
1991 3rd International Cytomegalovirus Workshop - Bologna
5th International Conference on Immunobiology and Prophylaxis of Herpesvirus Infections - St Petersberg FL
1992 IRBM Meeting on The Molecular Basis of Viral Latency - Rome
NIH Child Health and Human Development Workshop on Congenital CMV - Bethesda
First International Betaherpesvirus Symposium in Japan - Osaka
Banbury Conference on Molecular Mechanisms of Viral Latent Infections - Cold Spring Harbor
1993 Wallace Rowe Symposium - NIH, Bethesda
UCLA Symposium: Molecular Biology of Human Pathogenic Viruses - Lake Tahoe
4th International Cytomegalovirus Conference - Paris
6th International Conference on Immunobiology and Prophylaxis of Herpesvirus Infections - Hokkaido
1994 International Society for Antiviral Research - Charleston
Collaborative Antiviral Program - NIH, Bethesda
American Society for Microbiology Annual Meeting - Las Vegas
19th International Herpesvirus Workshop - Vancouver
4th International Meeting of the Canadian Bone Marrow Transplantation - Ottawa
1995 5th International Cytomegalovirus Workshop - Stockholm
20th International Herpesvirus Workshop - Groningen, Netherlands
7th International Conference on Immunobiology and Prophylaxis of Herpesvirus Infections - St. Petersberg, FL
1996 Glasgow Virology Workshop, Scotland
Consensus Symposium on Advances in Diagnosis, Treatment and Prophylaxis of CMV Infection - Sanibel Island, FL
21st International Herpesvirus Workshop - DeKalb, IL

1997 136th Society for General Microbiology General Meeting - Reading, United Kingdom
Animal Viruses Gordon Conference - Tilton, NH
8th International Conference on Immunobiology and Prophylaxis of Herpesvirus Infections - Mishima, Japan
Ocular Herpesvirus Research Workshop, Granlibakken, CA
37th Interscience Conference on Antimicrobial Agents and Chemotherapy - Toronto, Canada

1998 National Advisory Allergy and Infectious Diseases Council - Pathogen Genome Sequencing and Beyond, Bethesda, MD.

1999 Elkin Lecture, Emory University.
CMV Retinitis - 2nd Multidisciplinary Research Workshop, Yosemite, CA
7th International Cytomegalovirus Workshop, Keynote, Brighton, England
Robert H. Lurie Cancer Center Basic Science Colloquium, Chicago, IL
9th International Conference on Immunobiology and Prophylaxis of Herpesvirus Infections - Lucca, Italy
Danish Royal Biology Society lecture, Copenhagen

2000 Consensus Conference: Prevention of Post-Transfusion CMV in the Era of Universal Leukoreduction, Toronto
FASEB Microbial Pathogenesis Summer Conference, Aspen
3rd Symposium on Cytomegalovirus-related Immunopathology, Bertinoro, Italy
CDC Cytomegalovirus Vaccine Workshop, Altanta
University of Oklahoma Pfizer Visiting Professor in Infectious Diseases

2001 Keystone Conference: Control of Viral Latency and Persistence Keystone
Keystone Conference: Molecular Aspects of Viral Immunity
8th International Cytomegalovirus Workshop, Pacific Grove
Gordon Conference on Viruses and Cells, Lucca, Italy
26th International Herpesvirus Workshop, Regensburg, Germany
39th Infectious Disease Society of America Meeting, San Francisco
Inauguration Symposium - Virology Institute of Tubingen University
NIH NIAID Viral Mechanism of Immune Evasion Workshop, Annapolis
10th International Conference on Immunobiology and Prophylaxis of Herpesvirus Infections, Osaka.

2002 American Transplant Congress, Washington, DC
Foundation Juan March Viral Immunomodulation, Madrid
Trudeau Institute, Saranac Lake
International Joint Meeting on Cytokines (Cytokines 2002), Turin

2003 9th International Cytomegalovirus Workshop/1st International Betaherpesvirus Workshop - Maastricht
7th Symposium on Virus-Host Interactions, Mt Sinai School of Medicine

2004 Keystone Symposium, The Pathogen: Host Standoff – Taos
University of Chicago Symposium honoring Bernard Roizman.

2005 American Society for Microbiology Viral Evasion Workshop – Acapulco
10th International Cytomegalovirus Workshop/2nd International Betaherpesvirus Workshop – Williamsburg
European Society for Clinical Virology – Geneva

Immune Evasion Strategies of Human Pathogens & Celebration of the 40th Anniversary of the Salk Institute – Annecy
Banbury Conference on Early Events in Viral Infection - Cold Spring Harbor
Immunobiology and Immunoprophylaxis of Human Herpesvirus Infections – Osaka.

2006 Pathogen: Host Standoff Keystone Conference - Keystone
Society for General Microbiology General Meeting – Warwick
5th International Conference on HHV-6 & -7 - Barcelona
American Society for Microbiology General Meeting – Orlando
Closing Talk, XXXI International Herpesvirus Workshop – Seattle

2007 Virology Symposium, Duke University - Durham
Kaplan Symposium, University of Kentucky - Lexington
11th International CMV/3rd International Betaherpesvirus Workshop - Toulouse
XXXII International Herpesvirus Workshop – Asheville
Jamie McNew Lecture, University of Minnesota – Minneapolis.
SENS3 – Cambridge UK

2008 Hilleman Lecture – The University of Chicago
ICAAC-IDSA Joint Conference, Washington DC
University of Tubingen Dean's Research Colloquium

2009 Pediatric Infectious Disease Research Conference – St Judes
12th International Cytomegalovirus/4rd International Betaherpesvirus Workshop - Boston
Gordon Conference on Viruses & Cells – Il Ciocco
3rd World Congress Vaccines – Singapore
14th Immunoprophylaxis and Immunotherapy of Human Herpesvirus Infections – Kobe

2010 FASEB Virus Structure and Assembly Summer Research Conference
18th Euroconference on Apoptosis – Ghent, Belgium
Keynote - Nebraska Virology Center 10th Anniversary Symposium

2011 EBV Vaccine Meeting, NIH Vaccine Center - Bethesda
Keynote - 13th International Cytomegalovirus/5th International Betaherpesvirus Workshop – Nuremburg, Germany
Keynote - 36th International Herpesvirus Workshop – Gdansk, Poland
Keynote – Southeast IDeA Joint Program Meeting – New Orleans
15th Immunobiology of Herpesviruses Meeting – Venice, Italy

2012 Joint FDA-NIH-CDC Congenital CMV Workshop – NIH Bethesda
4th Australasian Vaccine and Immunotherapeutics Development Congress – Brisbane
2012 Helmholtz Center Symposium – Braunshweig
2012 Awaji International Forum on Infection and Immunity - Kobe
2012 Cell Death Gordon Research Conference – Il Ciocco
37th International Herpesvirus Workshop – Calgary
CMV2012 - Combined 14th Cytomegalovirus Workshop and 4th Congenital CMV Conference – San Francisco

2013 AACBNC Chair's Meeting – Puerto Rico
NCI Special Meeting on CMV in Glioblastoma – Bethesda

Great Lakes Transplant Immunology Forum – Atlanta
2014 Keystone Cell Death Meeting – Santa Fe
Keynote at the Virginia Regional Herpes Symposium – Norfolk
Keynote at the International Forum for Herpesviruses – Guangzhou
Waldthausen Symposium on Cytomegaloviruses
2015 Weller-Smith Oration at the 15th International Cytomegalovirus/7th International Betaherpesvirus Workshop and 5th Congenital CMV Disease Workshop – Brisbane, Australia
Nirit and Michael Shaoul Fellow Seminar at the University of Tel Aviv – Israel
Mechanisms of Microbial-Host Cell Manipulation - Erlangen, Germany
Cell Symposium on Cell Death and Immunity, Berkeley
2016 Keystone Conference on Persistent Virus Infection, Banff, Canada
VISTRIE Symposium, Braunschweig, Germany
Novarello Summer Course in Intrinsic and Innate Immunity, Novaro. Italy
Nebraska INBRE Retreat, Nebraska City
Nature Conference Innate Host Defense, Wuhan, China
2017 64th Society for Reproductive Investigation, Orlando
16th International Cytomegalovirus/8th International Betaherpesvirus Workshop and 6th Congenital CMV Disease Workshop – Noordwijkerhout, Netherlands;
Banbury Necroptosis Conference – Cold Spring Harbor Labs
2018 Keynote-Henle Lectureship, University of Pennsylvania Herpesvirus Symposium
Helmholtz Summer School on Infection Research - Wernigerode
Waldthausen Symposium on Cytomegalovirus in Aging
2019 EMBO Workshop on Pathogen Sensing and Innate Immunity – Oxford
44th International Herpesvirus Workshop – Knoxville (Invited Closing Keynote)
Italian Congress of Virology - Padua
European Cell Death Organization Meeting – Dresden
7th Chinese Herpesvirus Workshop – Shenzhen
2020 (Microbiology Society Annual Meeting – Edinburgh, *canceled*)
2021 A, B and Z: The Structure, Function and Genetics of Z-DNA and Z-RNA (virtual),
University of Iowa Mark Stinski Symposium – Iowa City
2022 Microbiology Society Annual Meeting – Belfast (postponed from 2020)
18th International Cytomegalovirus/8th Congenital CMV Disease Workshop – Cambridge (virtual)
EMBO Workshop on Cell Death in Host Defense – Crete
2023 Cologne Spring Meeting

Other Invited Seminars and Presentations:

1985 UC Los Angeles, Syntro, UC Irvine, DNAX Research Institute, Animal Cells and Viruses Gordon Conference, American Society of Virology
1986 University of Nevada, Dupont Research, Schering Research
1987 Chiron Corp., University of Chicago, University of Alabama, University of Kentucky, Scripps Clinic and Research Foundation, DNAX, UC San Francisco

1988 University of Rochester, Schering Research, Syntex Corp., University of Washington, University of Minnesota, Louisiana State University, NIH National Cooperative Vaccine Discovery Groups in AIDS Workshop, Gilead Sciences

1989 University of Chicago, Miles Laboratories, Schering Research

1990 University of British Columbia - Vancouver, SyStemix Corp., US Biochemical Corp., University of North Carolina, Research Triangle Virology Group, University of Ulm, University of Bologna, University of Washington, Genentech

1991 University of Missouri Medical Center-Columbia, University of Tennessee - Knoxville, Protein Design Labs, University of Turin, Schering Research, Irwin Memorial Blood Center, University of Ferrara, SmithKline Beecham Corp., BioMega, Jefferson Medical College

1992 UpJohn Corp., Louisiana State University – Baton Rouge, Linus Pauling Institute – Palo Alto, ICLAM Forum Lake Tahoe, International Herpesvirus Workshop - Edinburgh, University of Western Australia - Perth, University of Melbourne, Institute for Medical and Veterinary Sciences - Adelaide, Ribozyme Pharmaceuticals Corp.

1993 Michigan State University, Palo Alto Medical Foundation, 18th International Herpesvirus Workshop - Pittsburgh, UC San Francisco, University of Osaka, Royal Free Medical School - London, UC Davis, University of Washington

1994 University of Pennsylvania Medical School, Aviron Corp - Burlinghame, 19th International Herpesvirus Workshop - Vancouver, City of Hope Medical Center - Duarte, University of California - Irvine, Gilead Sciences - Foster City

1995 Northwestern University Medical School, McMaster University School of Medicine, University of Southern California School of Medicine, Harvard University School of Medicine, University of Colorado - Boulder, University of Gothenberg, Cambridge University, Smithkline Beecham Pharmaceuticals - Epson, Animal Viruses Gordon Conference - Tilton, 20th International Herpesvirus Workshop - Goningen, Scripps Research Institute, Smithkline Beecham Pharmaceuticals - King of Prussia.

1996 University of Glasgow, University of Bologna, Smithkline Beecham Biologicals - Belgium, St. Jude Children Research Hospital, University of Kansas, SmithKline Beecham Pharmaceuticals - King of Prussia.

1997 University of Nebraska, CMV Retinitis (A Multidisciplinary Workshop)-San Francisco, University of Tennessee-Knoxville, University of Taiwan, University of Pennsylvania, Oregon State University of the Health Sciences, UCSF Center for AIDS Research, University of British Columbia-Vancouver, University of Rijeka-Croatia, Robarts Research Institute-London, Ontario.

1998 St. Jude Children Research Hospital, Children's National Research Hospital, Ribozyme Pharmaceutical, Inc., Cytomegalovirus Latency Discussion Group, Keystone Symposium on Molecular Aspects of Viral Immunity, 23rd International Herpesvirus Workshop, UC Berkeley Program in Microbial Biology, University of Michigan, Parke-Davis Pharmaceuticals, Gladstone Institute for Virology, University of North Carolina.

1999 State University of New York at Buffalo, Northwestern University, University of Turin, University of Bologna, Baylor College of Medicine, University of Illinois School of Medicine-Chicago, Fox Chase Cancer Center, University of Munich.

2000 University of Massachusetts.
2001 University of California, Los Angeles; University of Iowa; Cleveland Clinic and Research Institute; University of Nebraska; University of North Carolina; University of Edmonton; UCSF-Gladstone Research Foundation; International Congress of Immunosuppression, San Diego; Northwestern University School of Medicine, Chicago.
2002 Vaccine Research Center of NIAID-NIH, Bethesda; ImmunoGen, Cambridge; Fred Hutchinson Cancer Research Center, Seattle; Ohio State University, Columbus; Ohio University, Athens; University of Sydney School of Medicine; ViroPharma, Exton; University of Padua, Italy.
2003 University of Maastricht, Netherlands; Imperial College School of Medicine, London; University of California, San Diego; University of Mainz, Germany; University of Pavia, Italy, Washington University, St. Louis; University of California – Irvine.
2004 Duke University Medical School, Louisiana State University Medical School – Shreveport, College of Medicine and Dentistry of NJ – Newark, Emory University – Atlanta, University of Pennsylvania School of Medicine – Philadelphia, Drexel University School of Medicine – Philadelphia, Stanford Symposium for Sam Karlin, University of North Carolina, Roizman 75th Birthday Symposium at the University of Chicago
2005 Stanford University Cardiovascular Research Program, Emory University – Atlanta, Berlex/Schering AG – Laguna Beach
2006 University of Tennessee – Knoxville, Southeast Regional Virology Conference – Atlanta, University of Tubingen, University of Padua, University of Croatia, Stinski Symposium at the University of Iowa, Harvard University School of Medicine – Boston, Children's Hospital National Research Center – Washington DC, Novartis Research – Cambridge, University College - London
2007 CDC – Atlanta, Georgia State University – Atlanta, Emory University School of Medicine – Atlanta, University of Minnesota school of Medicine – Minneapolis, University of Cambridge School of Medicine – Cambridge UK, University of Nebraska/Nebraska Center for Virology – Lincoln, Baylor College of Medicine – Houston.
2008 Louisiana State University – Baton Rouge, La Jolla Institute or Allergy & Immunology, University of California – Berkeley, Southeast Virology Conference – Atlanta, International Herpesvirus Workshop – Estoril, University of Munich, University of California Department of Medicine, Institute of Medicine National Vaccine Planning Meeting, CDC CMV Congenital Disease Conference
2009 University of South Florida – St Petersburg, University of Chicago, International Initiative for AIDS Vaccines, Emory Vaccine Center, MedImmune-Cambridge, Institute of Medical Sciences - University of Tokyo, Arizona State University Medical Center – Phoenix
2010 University of Washington, Fred Hutchinson Cancer Research Center, Southeast Regional Virology Conference - Atlanta
2011 Children's Hospital of Oakland Research Institute, Univ Pennsylvania – Philadelphia, ID Seminar - Emory University, HudsonAlpha Research Institute – Huntsville, Boehringer-Ingelheim – Laval, Montreal, GSK – Collegeville, PA,

University of Padova - Italy, University of Rijeka – Croatia, University of Pittsburgh, University of California – Irvine, GlaxoSmithkline – College Park

2012 NIH/NIAID Vaccine Research Center, University of Nevada – Reno, Louisiana State University Health Science Center – New Orleans, University of San Francisco, University of Sydney, University of Western Australia, University of Canberra, Institut Pasteur – Paris, NCI meeting on EBV-associated Burkitt's Lymphoma, Heinrich Pette Institute at the University of Hamburg, National Institute of Biomedical Innovation - Osaka

2013 University of Kyoto, GRC Conference on Virus and Cells– Il Ciocco, IHW – Grand Rapids, Emory/GA Tech Regenerative Medicine Group – Atlanta, Fox Chase Cancer Center - Philadelphia, University of Pennsylvania School of Medicine – Philadelphia, Jefferson University School of Medicine, Philadelphia

2014 Pfizer – Pearl River, NY, University of Massachusetts School of Medicine - Worchester, Tufts University School of Medicine - Boston, Institut Pasteur – Shanghai, SIOC of the Chinese Academy of Sciences – Shanghai, Wuhan University, Xiamen University, Roizman 85th Birthday Symposium at the University of Chicago, SUNY - Stony Brook, Merck – King of Prussia, GlaxoSmithKline – Collegeville, Charite University School of Veterinary Medicine – Berlin.

2015 Stanford University School of Medicine, Infectious Disease Research Institute - Seattle, University of Nebraska Virology Retreat, Walter and Eliza Hall Institute - Melbourne, University of Sydney, University of Tel Aviv, Weizmann Institute - Rehovat, Hadassa University School of Medicine - Jerusalem, University of Padua School of Medicine, 3rd European Seminar in Virology-Bertinoro, 39th International Herpesvirus Workshop - Boise, Georgia State University - Atlanta.

2016 MedImmune Translational Medicine - Mountain View; University of Mainz; University of Bologna; Harvard University - Cambridge; European Cell Death Organization - Barcelona; University of Croatia – Rijeka.

2017 University of Massachusetts Medical School – Worcester; Harvard Medical School – Boston; Beth Israel Deaconess Hospital – Boston; UC Berkeley; Aduro, Inc. – Berkeley; Viruses and Cells GRC – Il Ciocco, Italy.

2018 Cambridge University School of Medicine, University of Cardiff Medical School, Georgia State University, UC San Francisco, Tulane University – New Orleans, Northwestern University Medical School – Chicago, 42nd International Herpesvirus Workshop – Vancouver.

2019 18th International Cytomegalovirus Workshop – Birmingham, University of Texas, Southwestern – Dallas, National Institute for Biomedical Research – Beijing, Chinese Academy of Sciences – Beijing.

2021 Arizona State University, Biodesign Institute – Tempe (virtual), NIH NCI KSHV Vaccine Workshop (virtual); University of Illinois Medical Center – Chicago.

2022 University of Padua, University of Rijeka

Principal Organizer:

Major Meetings:

1991 XVI International Herpesvirus Workshop - Asilomar
2001 8th International Cytomegalovirus Workshop - Asilomar
2004 Keystone Symposium, The Pathogen:Host Standoff – Taos
2006 Keystone Symposium, The Pathogen:Host Standoff – Keystone
2008 Conference on Congenital CMV Disease – CDC
2012 Joint FDA-NIH-CDC Congenital CMV Workshop – NIH Bethesda

Workshops:

1987 West Coast Herpesvirus Workshop - Asilomar
1989 West Coast Herpesvirus Workshop - Asilomar
1990 West Coast Herpesvirus Workshop - Asilomar
1992 West Coast Herpesvirus Workshop - Asilomar
1993 West Coast Herpesvirus Workshop - Asilomar
1995 West Coast Herpesvirus Workshop - Reno
1998 Cytomegalovirus Latency Discussion Group - Tucson
2006 Transplacental Transmission and Control of Cytomegalovirus – Emory
2007 Transplacental Transmission and Control of Cytomegalovirus II – Emory
2008 Southeastern Regional Virology Conference – Atlanta
2012 Integrated FDA-NIH-CDC Congenital CMV Workshop – NIH Bethesda

International Organizing Committee:

1992 XVII International Herpesvirus Workshop - Edinburgh
1993 XVIII International Herpesvirus Workshop - Pittsburg
1993 4th International Cytomegalovirus Conference - Paris
1994 XIX International Herpesvirus Workshop - Vancouver
1995 5th International Cytomegalovirus Conference - Stockholm
1995 XX International Herpesvirus Workshop - Groningen
1997 6th International Cytomegalovirus Conference - Alabama
1997 XXII International Herpesvirus Workshop - San Diego
2001 XXVI International Herpesvirus Workshop – Regensburg
2003 9th International Cytomegalovirus Workshop/1st International Betaherpesvirus Workshop - Maastricht
2004 XXIX International Herpesvirus Workshop – Reno
2005 10th International Cytomegalovirus Workshop/2nd International Betaherpesvirus Workshop - Williamsburgh
2005 XXX International Herpesvirus Workshop – Turku
2006 XXXI International Herpesvirus Workshop – Seattle
2007 11th International Cytomegalovirus/3rd International Betaherpesvirus Workshop – Toulouse
2007 XXXII International Herpesvirus Workshop – Asheville
2008 XXXIII International Herpesvirus Workshop – Estoril
2008 Congenital CMV Conference – CDC campus Atlanta
2009 12th International Cytomegalovirus/4th International Betaherpesvirus Workshop – Boston

2011 XXXVI International Herpesvirus Workshop – Gdansk
2012 XXXVI International Herpesvirus Workshop – Calgary
2012 14th International Cytomegalovirus/6th International Betaherpesvirus Workshop – San Francisco and 4th Congenital CMV Disease Workshop
2013 XXXVII International Herpesvirus Workshop – Grand Rapids
2014 XXXVIII International Herpesvirus Workshop – Kobe
2016 XL International Herpesvirus Workshop – Madison
2017 16th International Cytomegalovirus/8th International Betaherpesvirus Workshop and 6th Congenital CMV Disease Workshop - Noordwijkerhout, Netherlands
2019 17th International Cytomegalovirus/7th Congenital CMV Disease Workshop – Birmingham
XLIV International Herpesvirus Workshop – Knoxville
2020 (XLV International Herpesvirus Workshop – Berlin – *cancelled*)
2022 18th International Cytomegalovirus/8th Congenital CMV Disease Workshop – Cambridge

PUBLICATIONS

Peer Reviewed Journal Articles (BOLD numbers indicate reports with high significance):

1. Mocarski, E.S. and M.F. Stinski (1979). Persistent infection of human fibroblast cells by human cytomegalovirus. J. Virol. 31:761-775.

2. Stinski, M.F., E.S. Mocarski, D.R. Thomsen and M. Urbanowski (1979). Membrane glycoproteins and antigens induced by cytomegalovirus. J. Gen. Virol. 43:119-129.

3. Stinski, M.F., E.S. Mocarski and D.R. Thomsen (1979). Some properties of cytomegalovirus DNA from standard and defective virions. J. Virol. 31:231-239.

4. Post, L.E., A.J. Conley, E.S. Mocarski and B. Roizman (1980). Cloning of reiterated and nonreiterated herpes simplex 1 sequences as BamHI fragments. Proc. Natl. Acad. Sci. USA 77:4201-4205.

5. Mocarski, E.S., L.E. Post and B. Roizman (1980). Molecular engineering of herpes simplex virus genome: Insertion of a second L-S junction into the genome causes additional genome inversions. Cell 22:243-255. PMID:6253078

6. Mocarski, E.S. and B. Roizman (1981). The site-specific inversion sequence of the herpes simplex virus genome: Domain and structural features. Proc. Natl. Acad. Sci. USA 78:7047-7051. PMID:6273905

7. Mocarski, E.S. and B. Roizman (1982). Herpesvirus dependent amplification and inversion of cell-associated viral thymidine kinase gene flanked by viral *a* sequences and linked to an origin of viral DNA replication. Proc. Natl. Acad. Sci. USA 79:5626- 5630. PMID:6291055

8. Mocarski, E.S. and B. Roizman (1982). The structure and function of the herpes simplex virus DNA termini: Implications regarding circularization, inversion and generation of virion DNA. Cell 31:89-97. PMID:6297756

9. Spaete, R.R. and E.S. Mocarski (1984). Trans-acting functions encoded by herpes simplex virus-1 recognize cis cleavage/packaging signals present on cytomegalovirus DNA. In F. Rapp (ed.) Herpesvirus, UCLA Symposium (new series) vol 21, Liss, Inc., New York.

10. Drew, W.L., E. Sweet, R. Minor, and E.S. Mocarski (1984). Multiple infections by cytomegalovirus in patients with acquired immunodeficiency syndrome: Documentation by Southern blot hybridization. J. Infect. Diseases 150:952-953. PMID:6094678

11. Mocarski, E.S., L. Deiss, and N. Frenkel (1985). Nucleotide sequence and structural features of a novel U_S-*a* junction present in a defective herpes simplex virus genome. J. Virol. 55:140-146.

12. Mocarski, E.S., L. Pereira, and N. Michael (1985). Precise localization of genes on large animal virus genomes: Use of λgtll and monoclonal antibodies to map a gene for a cytomegalovirus protein family. Proc. Natl. Acad. Sci. USA 82:1266-1270. PMID:2983334

13. Spaete, R.R. and E. S. Mocarski (1985). The *a* sequence of the cytomegalovirus genome functions as a cleavage/packaging signal for herpes simplex virus defective genomes. J. Virol. 54:817-824. PMID:2987533

14. Spaete, R.R., and E.S. Mocarski (1985). Regulation of cytomegalovirus gene expression: α and β promoters are trans-activated by viral functions in permissive human fibroblasts. J. Virol. 56:135-143. PMID:2993644

15. Geballe, A.P., F. L. Leach, and E.S. Mocarski (1986). Regulation of cytomegalovirus late gene expression: γ genes are controlled by posttranscriptional events. J. Virol. 57:864-874. PMID:3019554

16. Fiala, M.D., L. A. Cone, C-M. Chang, and E.S. Mocarski (1986). Cytomegalovirus viremia increases with progressive immune deficiency in patients infected with HTLV-III. AIDS Research 2:175-181.

17. Geballe, A.P., R.R. Spaete, and E.S. Mocarski (1986). A cis-acting element within the 5' leader of a cytomegalovirus β transcript determines kinetic class. Cell 46:865-872. PMID:3019554

18. Elias, P., M.E. O'Donnell, E.S. Mocarski and I.R. Lehman (1986). A DNA binding protein specific for an origin of replication of herpes simplex virus type 1. Proc. Natl. Acad. Sci. USA 83:6322-6326. PMID:3018724

19. Mocarski, E.S., A.C. Liu, and R.R. Spaete (1987). Structure and variability of the α sequence in the genome of human cytomegalovirus (Towne strain). J. Gen. Virol. 68:2223-2230. PMID:3039048

20. Spaete R.R. and E.S. Mocarski (1987). Insertion and deletion mutagenesis of the human cytomegalovirus genome. Proc. Natl. Acad Sci. USA 84:7213-7217. PMID:2823255

21. Kemble, G.W., A.L. McCormick, L. Pereira and E.S. Mocarski (1987). A cytomegalovirus protein with properties of herpes simplex virus ICP8: Partial purification of the polypeptide and map position of the gene. J. Virol. 61:3143-3151. PMID:3041036

22. Mocarski, E.S., W.C. Manning and J.M. Cherrington (1988). Recombinant cytomegalovirus-based expression vectors. In: Y. Gluzman and S.H. Hughes (eds.) Viral Vectors Cold Spring Harbor Press. pp. 78-84.

23. Ho, D. and E.S. Mocarski (1988). β-galactosidase as a marker in peripheral and neural tissues of the herpes simplex virus infected mouse. Virology 167:279-283. PMID:2847416

24. Geballe, A.P. and E.S. Mocarski (1988). Translational control of cytomegalovirus gene expression is mediated by upstream AUG codons. J. Virol. 62:3334-3340. PMID:2841486

25. Manning, W.C. and E.S. Mocarski (1988). Insertional mutagenesis of the murine cytomegalovirus genome: One prominent α gene (ie2) is dispensable for growth. Virology 167:477-484. PMID:2849236

26. Nasseri, M.F. and E.S. Mocarski (1988). The cleavage recognition signal is contained within sequences surrounding an *a-a* junction in herpes simplex virus DNA. Virology 167:25-30. PMID:2847414

27. Crute, J.J., E.S. Mocarski and I.R. Lehman (1988). A DNA helicase induced by herpes simplex virus type 1. Nucl. Acids Res. 16:6585-6596.

28. Mocarski, E.S., L. Pereira and A.L. McCormick (1988). Human cytomegalovirus ICP22, the product of the HWLF1 reading frame, is an early nuclear protein that is released from cells. J. Gen. Virol. 69:2613-2621.

29. Cherrington, J.M. and E.S. Mocarski (1989). Human cytomegalovirus ie1 transactivates the α promoter-enhancer via an 18-base-pair repeat element. J. Virol. 63:1435-1440. PMID: 2536844

30. Karlin, S., B.E. Blaisdell, E.S. Mocarski and V. Brendel (1989). A method to identify distinctive charge configurations in protein sequences, with application to human herpesvirus polypeptides. J. Mol. Biol. 205:164-178. PMID:2538622

31. Crute, J.J., T. Tsurimi, S.K. Weller, M.D. Challberg, E.S. Mocarski and I.R. Lehman (1989). The herpes simplex virus helicase-primase consists of three proteins encoded by the UL5, UL8 and UL52 genes. Proc. Natl. Acad. Sci. USA 86: 2186-2189.

32. Ripalti, A., M.P. Landini, E.S. Mocarski and M. La Placa (1989). Identification and preliminary use of recombinant λgtll fusion proteins in cytomegalovirus diagnosis. J. Gen. Virol. 70:1247-1251.

33. Leach, F.S. and E.S. Mocarski (1989). Regulation of cytomegalovirus late gene expression: Differential use of three start sites in the transcriptional activation of ICP36 gene expression. J. Virol 63:1783-1791. PMID:2538657

34. Kemble, G.W. and E.S. Mocarski (1989). A host cell protein binds to a highly conserved sequence element (pac-2) within the cytomegalovirus *a* sequence. J. Virol. 63:4715-4728. PMID:2552148

35. Ho, D. and E.S. Mocarski (1989). Herpes simplex virus latent RNA (LAT) is not required for latent infection in the mouse. Proc. Natl. Acad. Sci. USA 86:7596-7600. PMID:2552449

36. Sambucetti, L.C., J.M. Cherrington, G.W.G. Wilkinson and E.S. Mocarski (1989). NF-κB activation of the cytomegalovirus enhancer is mediated by a viral transactivator and by T cell stimulation. EMBO J. 8:4251-4258. PMID:2556267

37. Casareale, D., M. Fiala, C.M. Chang, L.A. Cone and E.S. Mocarski (1989). Cytomegalovirus enhances lysis of HIV-infected T lymphoblasts. Int. J. Cancer 44:124-130.

38. Cherrington, J.M., E. Khoury and E.S. Mocarski (1991). Human cytomegalovirus *ie*2 negatively regulates α gene expression via a short target sequence near the transcription start site. J. Virol. 65:887-896. PMID:1846203

39. Schachtel, G.A., P. Bucher, E.S. Mocarski, B.E. Blaisdell and S. Karlin (1991). Evidence for selective evolution in codon usage in conserved amino acid segments of human alphaherpesvirus proteins. J. Mol. Evol. 33:483-494. PMID:1663999

40. Stasiak, P.C. and E.S. Mocarski (1991). Transactivation of a cytomegalovirus γ gene promoter requires TRS1, a member of the US22 family. In M. P. Landini (ed.) Progress in Cytomegalovirus Research, Elsevier Science Publishers, Amsterdam. 63-79.

41. Ripalti, A. and E.S. Mocarski (1991). The products of human cytomegalovirus genes UL1-UL7, including gp48, are dispensable for growth in cell culture. In M. P. Landini (ed.) Progress in Cytomegalovirus Research, Elsevier Science Publishers, Amsterdam. 57-60.

42. Stasiak, P.C. and E.S. Mocarski (1992). Transactivation of the cytomegalovirus ICP36 gene promoter requires the α gene product TRS1 in addition to IE1 and IE2. J. Virol. 66:1050-1058. PMID:1370547

43. Maciejewski, J., E. Bruening, E. Mocarski, N. Young and S. C. St Jeor (1992). Infection of hematopoietic progenitor cells by human cytomegalovirus. Blood 187:170-178. PMID:1377049

44. Dormitzer, P.R., D.Y. Ho, E.R. Mackow, E.S. Mocarski and H. B. Greenberg (1992). Neutralizing epitopes on herpes simplex virus-1-expressed rotavirus VP7 are dependent on coexpression of other rotavirus proteins. Virology 187:18-32. PMID:1371025

45. Dutch, R. E., R. C. Bruckner, E. S. Mocarski and I. R. Lehman (1992). Herpes simplex virus type 1 recombination: Role of DNA replication and *a* sequences. J. Virol. 66:277-285. PMID:1309247

46. Masse, M.J., S. Karlin, G.A. Schachtel and E.S. Mocarski (1992). Human cytomegalovirus origin of DNA replication (oriLyt) resides within a highly complex repetitive region. Proc. Natl. Acad. Sci. USA 89:5246-5250. PMID:1319057

47. Manning, W.C., C.A. Stoddart, L.A. Lagenaur, G.B. Abenes and E.S. Mocarski (1992). Cytomegalovirus determinant of replication in salivary glands. J. Virol. 66:3794-3802. PMID:1316482

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49. Mocarski, E.S., M. Bonyhadi, S. Salimi, J.M. McCune and H. Kaneshima (1993) Human cytomegalovirus in the SCID-hu mouse: Thymic epithelial cells are prominent targets of viral infection. Proc. Natl. Acad. Sci. USA 90:104-108. PMID:7678330

50. Ho, D.Y., E.S. Mocarski and R. Sapolsky (1993). Altering central nervous system physiology with a defective herpesvirus vector expressing the glucose transporter gene. Proc. Natl. Acad. Sci. USA 90:3655-3659. PMID:8386379

51. Cardin, R.D., J.M. Boname, G.B. Abenes, S.A. Jennings and E.S. Mocarski (1993). Reactivation of murine cytomegalovirus from latency. In S. Michelson and S. A. Plotkin (ed.) Multidisciplinary Approaches to Understanding Cytomegalovirus Disease, Elsevier, Amsterdam. pp. 65-74.

52. Boname, J.M., L.A. Lagenaur and E.S. Mocarski (1994). Murine cytomegalovirus genes influencing virus growth and tropism for salivary gland. In Y. Becker and G. Darai (ed.) Frontiers of Virology, Vol. 3, Springer-Verlag, Heidelberg. pp. 315-328

53. Karlin, S., E.S. Mocarski and G.A. Schachtel (1994). Molecular evolution of herpesviruses: Genomic and protein sequence comparisons. J. Virol. 68:1886-1902. PMID:8107249

54. Vieira, J., H.E. Farrell, W.D. Rawlinson, and E.S. Mocarski (1994). Genes in the *Hin*dIII J fragment of the murine cytomegalovirus genome are dispensable for growth in cultured cells: Insertion mutagenesis with an *lac*Z/*gpt* cassette. J. Virol. 68:4837-4846. PMID:8035482

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Videos:

(can be downloaded from www.YouTube.com)

Science in the Courtroom Program 1: Core Concepts of Microbiology

Stephen J. Breyer; Edward S. Mocarski Jr. Federal Judicial Television Network (FJTN) Federal Judicial Center January 10, 2001 4046-V/01 (DVD) (40 minutes)

This is the first of six programs in the FJC's Science in the Courtroom series. After Justice Stephen Breyer introduces the series, Professor Edward S. Mocarski, Jr., of Stanford University Medical School, provides an overview of key concepts in basic microbiology. He explains the universality of the genetic code (i.e., how genes are made up of molecules of DNA), how DNA's four bases are common to all organisms, and how the arrangement of those bases dictates the differences in organisms. Professor Mocarski describes the scientific understanding of how DNA can be copied or reproduced by an intermediary, RNA, and "expressed" into proteins that carry out the work of life. This is the basic "molecular dogma" used by scientists to understand how to manipulate genes in processes of gene therapy, genetic engineering, and gene cloning.

Science in the Courtroom Program 2: Recombinant DNA and Gene Cloning

Edward S. Mocarski Jr. Federal Judicial Television Network (FJTN) Federal Judicial Center January 10, 2001 4047-V/01 (DVD) (36 minutes)

This is the second of six programs in the FJC's Science in the Courtroom series. Professor Edward S. Mocarski, Jr., of Stanford University Medical School, builds upon his lecture in Part One of the series (Core Concepts of Microbiology) by explaining the basic recombinant DNA and gene-cloning methods used in the field of biotechnology. Mocarski explains how the universality of the genetic code makes it possible for scientists to recombine DNA, that is, take DNA from one organism and move it into another. He also explains how recombinant DNA concepts are used in the "expression" of human proteins into bacteria, a process in which a human coding sequence is taken and inserted into a bacterial context, allowing the bacteria to produce abundant supplies of a "foreign protein" (foreign to the bacteria) that can be of commercial and therapeutic use.